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Prevalence of Hepatitis E Virus (HEV) Infection in Wild Boars (*Sus scrofa leucomystax*) and Pigs in Gunma Prefecture, Japan

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ABSTRACT. The prevalence of hepatitis E virus (HEV) infection in wild boars and pigs in Gunma Prefecture, Japan, was serologically and genetically examined. The positive detection rates of anti-HEV IgG and HEV RNA in the wild boars were 4.5% (4/89) and 1.1% (1/89), whereas those in the pigs were 74.6% (126/169) and 1.8% (3/169), respectively. The positive rates of anti-HEV IgG and HEV RNA on the 17 pig farms in the present study ranged from 20% to 100%, respectively. One male wild boar approximately 5 years of age was positive for HEV RNA but was negative for anti-HEV IgG. Three pigs from 2 farms were positive for HEV RNA; 2 of these pigs were negative for HEV IgG, and the other was positive. A phylogenetic analysis revealed that all of the HEV ORF1 genes detected in the present study belonged to genotype III. In Gunma Prefecture, HEV is highly prevalent and widespread, and uncooked wild boar and pig meat may have the potential to transmit HEV to humans.

KEY WORDS: hepatitis E virus, Japan, swine, wild boar.

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Hepatitis E virus (HEV), which belongs to the genus *Hepevirus*, is the causative agent of hepatitis E. Hepatitis E infection has been found in many developing countries in Asia, Africa and Latin America, where the disease is an important public health concern [15]. HEV is primarily transmitted by the fecal-oral route such as in waterborne epidemics.

Recent studies have suggested that HEV is divided into 4 genotypes designated as G I, G II, G III, and G IV [17]. The HEV infections in Asia and Africa are mainly caused by G I, and the majority of the GII infection have been reported in Mexico and Nigeria. On the other hand, only a single case of infection with GIII or GIV has been described in the United States, European counties, Argentina, Taiwan and China [17, 21, 22]. In Japan, most imported cases with G I have derived from epidemic areas such as Asia and Africa [2]; however, G III or G IV has also been detected in acute hepatitis patients who have never traveled to HEV epidemic areas [6, 8, 13, 14, 20, 21, 24, 29]. These patients often have a history of consuming uncooked wild boar (*Sus scrofa leucomystax*) and sika deer (*Cervus nippon*) meat and liver [5, 27, 28]. Also, HEV strains belonging to G I, G III or G IV have been detected in Japanese patients with sporadic acute or fulminate hepatitis E [8, 9, 19–22, 24, 31]. In addition, Yazaki *et al.* [31] reported that HEV RNA has been detected in 2% (7/363 packages) of sold pig liver on the market by reverse transcription-polymerase chain reaction (RT-PCR).

In Japan, it has been suggested that the transmission route of HEV remains unclear in approximately 60% of infected patients [1]; zoonotic food-borne transmissions account for 30%, imported infection accounts for 8% and blood transfusion is responsible for 2%. In Gunma Prefecture, Japan, approximately 3,000 wild boars are annually slaughtered for meat [unpublished data], and the number of breeding pigs in the prefecture was approximately 6 million in 2005. According to the Gunma Prefectural Statistics Report (http://www.pref.gunma.jp/cts/PortalServlet?DISPLAY_ID=DIRECT&NEXT_DISPLAY_ID=U000004&CONTENTS_ID=43375), Gunma Prefecture is one of the major pork-producing areas in Japan. However, to the best of our knowledge, there have been no reports on the prevalence of HEV infection in wild boars and pigs in the prefecture to date. Here in, we report the seroprevalence of anti-HEV IgG detected by enzyme-linked immunosorbent assay (ELISA) and HEV RNA by RT-PCR among wild boars and pigs in Gunma Prefecture, Japan.

MATERIALS AND METHODS

Samples: From September 2004 to March 2006, blood samples from 89 wild boars were kindly provided by hunters, and these samples were placed in sterile tubes, stored at approximately 4°C and sent to the laboratory within 12 hr. The ages of the wild boars were estimated by the hunters. From September to December 2004, we collected 169 pig blood samples from 17 pig farms during viscera inspections

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at G slaughterhouse in Gunma Prefecture, with 9 to 10 samples obtained from each farm. All pigs were approximately 6 months old. The blood samples were placed in sterile tubes, stored at approximately 4°C and sent to the laboratory within 3 hr. All blood samples were centrifuged at 1,900 × *g* for 20 min, and the serum was stored at –20°C until analyses.

Serologic analysis: Anti-HEV IgG was measured by ELISA as previously described with some modifications [4]. The antigen used in the ELISA was HEV-like particles composed of a truncated open reading frame 2 (ORF2) protein of genotype I HEV expressed by a recombinant baculovirus in insect cells and was suspended with 0.5 M carbonate buffer (pH 12.5) at a concentration of 1 μg/ml [3]. The antigen solution (100 μl) was added to duplicate wells of 96-well microplates (Sumiron ELISA plate type H, Sumitomo Bakelite, Tokyo, Japan). After washing with phosphate buffered saline containing 0.05% of tween-20 (PBST), the wells were coated with 5% skim milk in PBST for 1 hr at room temperature and then incubated with 100 μl of serum samples at a dilution of 1:200 in 1% skim milk in PBST for 1 hr at room temperature. The wells were washed with PBST 3 times, and the bound IgG antibodies were probed with peroxidase-labeled goat anti-swine IgG antibodies (heavy plus light chain; Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.). After washing 3 times with PBST, 100 μl of substrate, for wild boar samples, TMB HRP Microwell substrate, Bio FX Laboratories, MD, USA; for swine samples, 200 μM of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), Sigma, St. Louis, MO, U.S.A.) was added, and the plates were incubated for 10 min (for wild boar samples) or 30 min (for swine samples) at room temperature. Following the incubation period, 100 μl of stop solutions was added to the plates. The density at 450 nm (wild boar samples) or 415 nm (swine samples) was measured using an automatic ELISA reader (Benchmark Plus, BioRad, U.S.A.). A sample was considered positive for anti-HEV IgG when the average of OD value was greater than the cut-off value. To determine the cut-off value of the IgG, each of the 10 samples that had the lowest OD values and were negative in the western blot analysis were used as negative sera. In the present study, ODs of 2.597 and 0.197, which were calculated as three standard deviations above the mean values for the wild boar and swine negative controls, respectively, were used as the tentative cut-off values for each sample.

Extraction of RNA and reverse transcription polymerase chain reaction: Frozen serum samples were thawed at room temperature and then centrifuged at 3,000 × *g* at 4°C for 30 min, and the supernatants were then used for RT-PCR and sequence analysis. Total RNA was extracted from 140 μl of the re-centrifuged serum using a QIAamp Viral RNA Mini kit (Qiagen, MD, U.S.A.). The extracted RNA was then suspended in 60 μl of DNase/RNase-free water and treated with 5 U of DNase I (Takara, Tokyo, Japan). To amplify the 326- nucleotide region from open reading frame 1 (ORF1) by RT-PCR, we used genotype-specific primers as previously described [21]. The amplified DNA fragment was separated by electrophoresis on a 3% agarose gel, and the DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen). The nucleotide sequence was determined using an automated DNA sequencer (ABI PRISM™ 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, U.S.A.) using a Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems). Nucleotide sequences of the partial ORF1 of HEV (positions 124 to 449: 326 bp) were analyzed phylogenetically using CLUSTALW on the DNA database of Japan (DDBJ) homepage (http://hypernig.nig.ac.jp/homology/clustalw-e.shtml) and TreeExplorer (Version 2.12; http://evolgen.biol.metro-u.ac.jp/TE/). Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenic trees were constructed using the neighbor-joining (NJ) method [16]. The reliability of the trees was estimated using 1000 bootstrap replications.

Statistical analysis: The chi-square test with Yates' continuity correction was used to compare the positive detection rates of anti-HEV IgG between male and female wild boars. Differences were considered significant when the p value was less than 0.05.

RESULTS

Prevalence of HEV infection in wild boars in Gunma Prefecture: Anti-HEV IgG was detected in 4 (4.5%) of the 89 wild boars (Table 1). No significant difference was found between for the male (2.9%; 1/35) and female (6.7%; 3/45) wild boars (chi-square test with Yates' continuity correction, p=0.7960). HEV was detected in only 1 wild boar (WBG06–01), giving a 1.1% (1/89) positive rate.

Prevalence of HEV infection in slaughtered pigs in Gunma Prefecture: Anti-HEV IgG was detected in 126 (74.6%) of the 169 slaughtered pigs (Table 2). The positive rates among the individual 17 pig farms varied from 20% to 100%. HEV RNA was detected in 1.8% (3/169) of the pigs, 1 from farm M(PG05–03) and 2 from farm H (PG05–01 and PG02–02), and the positive rates of anti-HEV IgG for these farms were 60% and 89%, respectively.

Information on HEV RNA-positive animals: We detected HEV RNA in one wild boar and three pigs. The wild boar (WBG06–01; male; body weight of about 80 kg) was estimated to be approximately 5 years of age by the hunters and was negative for anti-HEV IgG. Of the 3 pigs, 2 (PG05–01 and PG05–02) were from farm H, and the other (PG05–03) was from farm M. Farms E and L are located in the center of Gunma Prefecture and have no history of contact with wild boars. Of these 3 pigs, 2 (PG05–01 and PG05–03) were negative and 1 (PG05–02) was positive for anti-HEV IgG.

Phylogenetic analysis of the HEV isolates based on the sequences of open reading frame 1: The phylogenetic tree based on the ORF1 gene in HEV detected in Japan and other countries is shown in Fig. 1. The strains were divided into 4 genotypes as described in a previous report [17]. All 4

Table 1. Detection of anti HEV-IgG and HEV RNA in wild boars

Age (months)	Sex	Number of samples	IgG positive samples (%)	HEV RNA detection (%)
< 12	Male	4	0 (0)	0 (0)
	Female	7	0 (0)	0 (0)
13–24	Male	8	0 (0)	0 (0)
	Female	3	0 (0)	0 (0)
25–36	Male	4	1 (25.0)	0 (0)
	Female	10	0 (0)	0 (0)
37–48	Male	6	0 (0)	0 (0)
	Female	7	0 (0)	0 (0)
49–62	Male	1	0 (0)	1[a](100)
	Female	9	1 (11.1)	0 (0)
>62	Male	5	0 (0)	0 (0)
	Female	6	1 (16.7)	0 (0)
Unknown	Male	7	0 (0)	0 (0)
	Female	3	1 (33.3)	0 (0)
	No record	9	0 (0)	0 (0)
Subtotal	Male	35	1 (2.9)	1 (2.9)
	Female	45	3 (6.7)	0 (0)
	No record	9	0 (0)	0 (0)
Total		89	4 (4.5)	1 (1.1)

a) Sample number: WBG06–01.

Table 2. Detection of anti HEV-IgG and HEV RNA in 17 pig farms

Farm	Number of samples	IgG positive samples (%)	HEV RNA detection (%)
A	10	10 (100)	0 (0)
B	10	10 (100)	0 (0)
C	10	10 (100)	0 (0)
D	10	10 (100)	0 (0)
E	10	10 (100)	0 (0)
F	10	9 (90)	0 (0)
G	10	9 (90)	0 (0)
H	9	8 (88.9)	2[a](22.2)
I	10	8 (80)	0 (0)
J	10	7 (80)	0 (0)
K	10	7 (70)	0 (0)
L	10	6 (70)	0 (0)
M	10	6 (60)	1[b](10)
N	10	5 (50)	0 (0)
O	10	5 (50)	0 (0)
P	10	4 (40)	0 (0)
Q	10	2 (20)	0 (0)
Total	169	126 (74.6)	3 (1.8)

a) Sample number: PG05–01 and PG05–02.
b) Sample number: PG05–03.

strains detected in the present study were classified into genotype III, which includes several genotypes of Japanese domestic animals previously reported [8, 11, 12, 20, 23, 28, 31]. The sequences of the 2 pigs (PG05–01 and PG05–02) from farm H were identical (AB362371 and AB362372) but were different from that for farm M by approximately 0.11, while the distances of the wild boar sequence (AB362374) from the sequences of boars from farms H and M were 0.1 and 0.07, respectively.

DISCUSSION

Epidemiological studies have reported that HEV infection is prevalent among wild boars [5, 12, 26, 30] and pigs [10, 25] and have suggested that consumption of the meat and liver of these animals is a risk in terms of HEV infection in Japan [5, 26, 30]. In the present study, the positive rates of anti-HEV IgG and HEV RNA (genotype III) in the wild boars were 4.5% (4/89) and 1.1% (1/89), respectively. The positive detection rates showed no relationship with the age of the animals. Sonoda *et al.* [18] reported that anti-HEV IgG is present in 8.6% (3/35) of wild boars and that HEV RNA genotype III has been detected in a 60-kg male wild boar (2.9%, 1/35) that was negative for anti-HEV IgG (presumed to be approximately 2 years of age). In other study in Japan, Michitaka *et al.* [7] reported a positive rate of anti-HEV IgG in wild boars of 25.5% (100/392), and 3.1% (12/392) of the wild boars in their study were positive for the HEV RNA genotype III. In the present study, although the seroprevalence of HEV infection in the wild boar was considerably lower than in previous reports, some of the animals in Japan are infected with GIII and may potentially serve as a source of infection in humans.

The prevalence of anti-HEV IgG in pigs depends on the age of the animals, and HEV RNA has been detected in 2- to 4-month-old pigs and less commonly in older pigs [10, 14, 23, 25]. Takahashi *et al.* [23, 25] reported detection rates of anti-HEV IgG in 6-month-old pigs that ranged from 73.5% (100/136) to 90.4% (226/250), with no HEV RNA detection from any prefecture examined in Japan to date. Although the positive rates of anti-HEV IgG in the present study were similar to those in previous reports, HEV RNA (genotype III) was detected in 1.8% (3/169) of the pig serum samples, and this suggests that HEV genotype III is highly prevalent and widely distributed in pigs. Thus, it is highly possible that pigs are a source of HEV infection in humans. A nationwide campaign prohibiting consumption of uncooked liver and meat from wild boars and pigs should be implemented to prevent HEV infection in humans.

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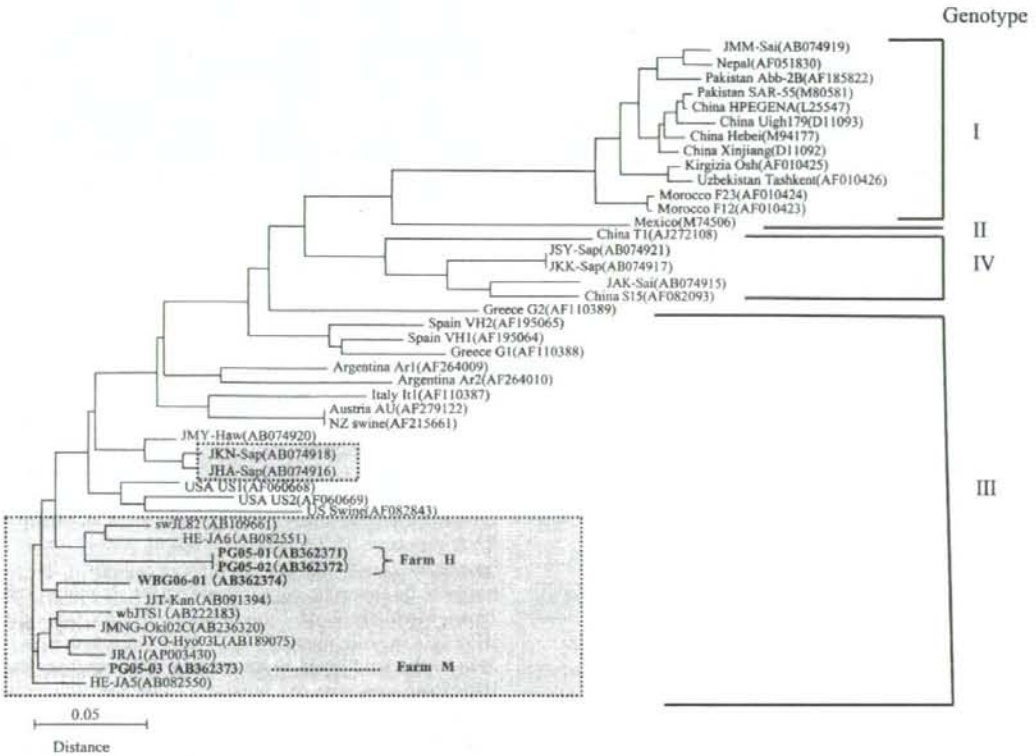

Fig. 1. Phylogenetic trees on the basis of 326 nt of the ORF1 region constructed by the neighbor-joining method [16]. The HEV strains from one wild boar and three pigs from farms are shown in bold type. In addition, genotype III strains reported in previous studies in Japan are indicated by gray boxes. The GenBank accession numbers of the identified strains are enclosed in parentheses.

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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

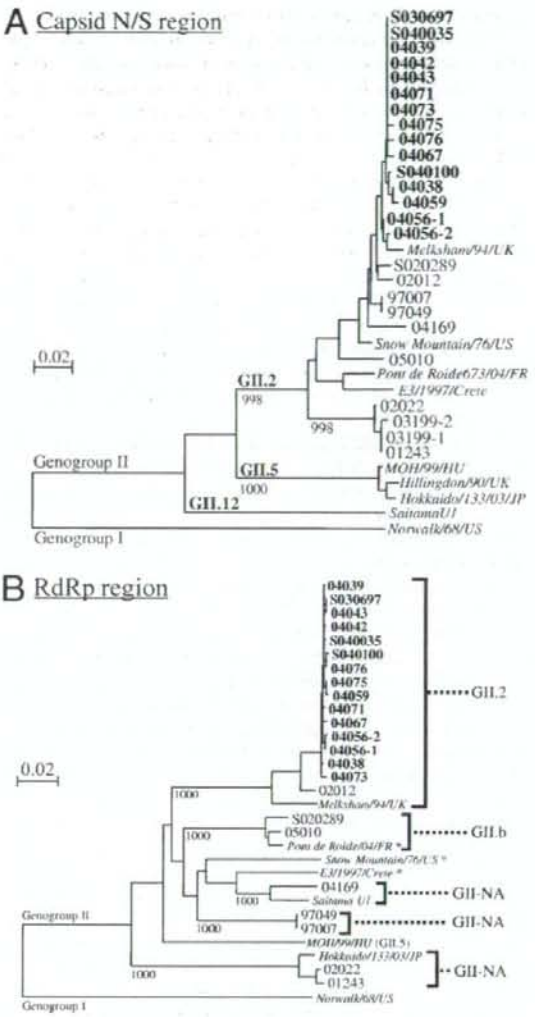

FIG. 1. Phylogenetic analysis of the capsid N/S (278 nucleotides) (A) and the partial RdRp (B) regions of the GII.2-cap strains detected in Osaka City. The GII.2-cap strains detected between March and May 2004 (04spring strains) are represented in boldface. Reference strains of NoV used in this study 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.

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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Genetic Analysis of the Capsid Gene of Genotype GII.2 Noroviruses▽

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Noroviruses (NoVs) are considered to be a major cause of acute nonbacterial gastroenteritis in humans. The NoV genus is genetically diverse, and genotype GII.4 has been most commonly identified worldwide in recent years. In this study we analyzed the complete capsid gene of NoV strains belonging to the less prevalent genotype GII.2. We compared a total of 36 complete capsid sequences of GII.2 sequences obtained from the GenBank ($n = 5$) and from outbreaks or sporadic cases that occurred in The Netherlands ($n = 10$) and in Osaka City, Japan ($n = 21$), between 1976 and 2005. Alignment of all capsid sequences did not show fixation of amino acid substitutions over time as an indication for genetic drift. In contrast, when strains previously recognized as recombinants were excluded from the alignment, genetic drift was observed. Substitutions were found at five informative sites (two in the P1 subdomain and three in the P2 subdomain), segregating strains into five genetic groups (1994 to 1997, 1999 to 2000, 2001 to 2003, 2004, and 2005). Only one amino acid position changed consistently between each group (position 345). Homology modeling of the GII.2 capsid protein showed that the five amino acids were located on the surface of the capsid and close to each other at the interface of two monomers. The data suggest that these changes were induced by selective pressure, driving virus evolution. Remarkably, this was observed only for nonrecombinant genomes, suggesting differences in behavior with recombinant strains.

Noroviruses (NoVs) are an important cause of acute nonbacterial gastroenteritis in adults and children worldwide (13). NoVs are members of the family *Caliciviridae*, having a positive-sense single-stranded RNA genome. Their genome is organized into three open reading frames (ORFs). ORF1 encodes nonstructural proteins including the RNA-dependent RNA polymerase (RdRp), ORF2 encodes a major structural capsid protein including a shell (S) domain and a protruding (P) domain, and ORF3 encodes a minor structural protein (13, 18, 41). The S domain forms the inner part (shell) of the viral capsid, and the P domain forms the arch-like structures that protrude from the virion. The P domain is further divided into P1 and P2 subdomains that correspond to the sides and the top of the arch-like capsomeres, respectively (13, 31).

Based on the genetic analysis of the RdRp and capsid regions, human NoVs can be divided into three genogroups (Gs), GI, GII, and GIV (2, 14, 39), which further segregate into distinct lineages called genotypes (2, 20, 36, 37). Recently, Kageyama et al. (20) proposed that at least 31 genotypes could be distinguished within GI and GII. The GII.4 genotype, which is represented by the Lordsdale/93/UK strain, has been the most commonly identified genotype worldwide in recent years. Genetic characterization of strains belonging to this genotype have shown a sequence of variants that have arisen over time, suggesting that rapid genetic evolution of GII.4 NoVs may in part explain their successful spread and impact on people of all ages (5, 9, 27, 29, 30, 33, 40).

Viruses belonging to other NoV genotypes are found less consistently, causing sporadic outbreaks or temporary epidemics in a limited geographic region or time period (5, 17, 23, 26). As a result, far less is known about the population structure of these variants (28). The genetic analysis of other genotype NoVs excluding GII.4 will improve our understanding of genetic evolution and its relevance for the epidemiology of NoVs.

During the spring of 2004, an epidemic of GII.2 NoV (which is represented by the Melksham/94/UK strain [Melksham]) occurred in Osaka City, Japan. Our previous study of this regional epidemic described the molecular epidemiology of these GII.2 strains (17). Here, we describe the genetic characterization of GII.2 strains from those outbreaks in comparison with viruses detected over a 12-year period in the GenBank, The Netherlands, and Japan.

MATERIALS AND METHODS

GII.2 strains. A total of 36 NoV strains that had been characterized as GII.2 genotype were used for this study (Table 1). The capsid sequence data for five GII.2 strains were obtained from the GenBank. Of these, the Melksham strain and the Chesterfield/434/1997/US strain (11, 29) have been characterized as belonging to the GII.2 genotype on the basis of RdRp as well as capsid regions. The Snow Mountain/76/US strain has been characterized as a recombinant NoV, with a distinct (non-GII.2) RdRp region and a GII.2 capsid region (4, 16). The other two strains from the GenBank (Ina/02/JP and Buds/02/US) were characterized as GII.2 genotype on the basis of the capsid region, but their sequences of the RdRp region were unknown. The capsid sequence data of Buds/02/US strain lacked the first 6 nucleotides (nt) from the 5′ end of the capsid gene. Twenty-one GII.2 strains were obtained from outbreaks or sporadic cases detected in Osaka City, Japan, between April 1996 and March 2005. These were 21

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TABLE 1. GII.2 NoV strains used in this study

Source	Strain (abbreviation)[a]	RdRp sequence type[b]	Accession no.	Note[c]
GenBank	Snow Mountain/76/US (SM)	GII-NA	AY134748	
	Melksham/94/UK (Melksham)	GII.2	X81879	
	Chesterfield/434/97/US (CF434)	GII.2	AY054300	
	Ina/02/JP (Ina)	Unknown	AB195225	
	Buds02/US (Buds)	Unknown	AY660568	
Osaka City, Japan	OC97049/97/JP (97049)	GII-NA	AB279553	O
	OC01243/01/JP (01243)	GII-NA	AB279554	O
	OC02012/02/JP (02012)	GII.2	AB279555	O
	OC02022/02/JP (02022)	GII-NA	AB279556	O
	OCS020289/02/JP (S020289)	GII.b	AB279570	S
	OC04038/04/JP (04038)[d]	GII.2	AB279557	O*
	OC04042/04/JP (04042)[d]	GII.2	AB279558	O*
	OC04043/04/JP (04043)[d]	GII.2	AB279559	O*
	OCS030697/04/JP (S030697)[d]	GII.2	AB279571	S*
	OC04056-1/04/JP (04056-1)[d]	GII.2	AB279560	O*
	OC04056-2/04/JP (04056-2)[d]	GII.2	AB279561	O*
	OC04059/04/JP (04059)	GII.2	AB279562	O*
	OCS040035/04/JP (S040035)	GII.2	AB279572	S*
	OC04067/04/JP (04067)[d]	GII.2	AB279563	O*
	OC04071/04/JP (04071)	GII.2	AB279564	O*
	OC04073/04/JP (04073)	GII.2	AB279565	O*
	OC04075/04/JP (04075)	GII.2	AB279566	O*
	OC04076/04/JP (04076)[d]	GII.2	AB279567	O*
	OCS040100/04/JP (S040100)	GII.2	AB279573	S*
	OC04169/04/JP (04169)	GII-NA	AB279568	O
	OC05010/05/JP (05010)	GII.b	AB279569	O
The Netherlands	Sensor99-191/99/NL (S99-191)	GII.2	AB281081	S
	OB0037-246/00/NL (OB0037)	GII.2	AB281082	O
	OB0048-318/00/NL (OB0048)	GII.2	AB281083	S
	OB0115-195/01/NL (OB0115)	GII.2	AB281084	O
	EP0125-006/01/NL (EP0125)	GII.2	AB281085	O
	EP0207-001/02/NL (EP0207)	GII.2	AB281086	O
	EP0239-001/02/NL (EP0239)	GII.2	AB281087	O
	OB0371-459/03/NL (OB0371)	GII.2	AB281088	O
	OB0528-158/05NL (OB0528)	GII.2	AB281089	O
	OB0587-470/05/NL (OB0587)	GII.2	AB281090	O

[a] NoV strains are arranged in chronology of detection from top (oldest) to bottom (most recent) for each source.
[b] NA, not assigned.
[c] O, outbreak; S, sporadic case; *, spring epidemic in 2004.
[d] These strains have identical amino acid sequences in the complete capsid gene and only one strain (OC04038/04/JP) has been used for long-term genetic analysis.

of the 23 GII.2 strains identified during a 9-year study period out of a total of 238 outbreaks and 200 sporadic cases of NoV infection. From the genetic analysis across the junction between the RdRp and the capsid regions, 6 of these 21 GII.2 strains have been characterized as recombinants, which have non-GII.2 RdRp regions and GII.2 capsid regions (Fig. 1) (17).

The strains from The Netherlands were collected from a 12-year study period. Between 1994 and 2005, GII.2 NoVs were detected in 13 (1.7%) out of 745 NoV-associated outbreaks and three sporadic cases in The Netherlands. Initially, these GII.2 NoVs were characterized by the comparison of sequences in the RdRp region (Fig. 1). The detection method and criteria for genotyping at the RdRp region have been previously described (8, 36). The complete capsid genes of 10 strains from eight outbreaks and two sporadic cases were amplified by reverse transcription-PCR (RT-PCR) and were used for this analysis.

Amplification and sequencing for the complete capsid gene of GII.2 strains. Viral RNA was extracted from stool suspensions by using a QIAamp viral RNA Mini kit (Qiagen, Hilden, Germany). RT-PCR was carried out with the reaction mixtures and enzymes as previously described (8). RT was performed at 42°C for 2 to 3 h with reverse primer, N235Rex (17), and enzyme was inactivated at 95°C for 5 min. PCR was performed using several pairs of PCR primers (Table 2) with a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) under the following conditions: denaturation at 95°C for 1 min; 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 1 min; and a final cycle of incubation at 72°C for 5 min. When a PCR failed to produce strong products, we performed nested PCR. The amplified fragments were sequenced directly with a Big Dye terminator cycle sequencing kit and ABI 3700 sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequences were determined in both orientations using the PCR primers. DNA sequences were edited using SeqManII (DNAStar Inc., Konstanz, Germany).

Sequence analysis. Nucleotide or amino acid sequence alignments were performed with BioEdit (version 7.052) (15), Clustal X (version 1.81) (35), or MUSCLE (version 3.51) (10). The extraction of the informative sites from nucleotide or amino acid sequence alignments was performed with ProSeq (version 2.91) (12). The rate of change for different domains was compared using chi-square statistics. In this analysis, a site was designated as an informative site when at least two strains had an identical amino acid in the alignment that differed from the other sequences. A phylogenetic tree with 1,000 bootstrap replications was constructed by the neighbor-joining method, and the genetic distances were calculated according to the Kimura two-parameter method (21). We performed additional phylogenetic analysis by the Bayesian method using MrBayes (version 3.1.2) (32). Location of specific domains of the GII.2 NoV capsid gene was done according to Chen et al. (7). For computational predictions of the structure of the GII.2 NoV capsid protein, we used the X-ray crystal structures of the capsid protein of Norwalk/68/US (GI.1 genotype, Protein Data Bank identifier [PDB ID] 1IHM, consisting of a complete trimer) (31) and VA387/98/US (GII.4 genotype, PDB ID 2OBR, consisting of only a monomeric P domain) (6) as templates to build homology models. The sequence alignments for the structure and the three-dimensional (3D) models for GII.2 NoV capsid proteins were made by using the WHAT IF program (38) and the 3D-Jigsaw (3)

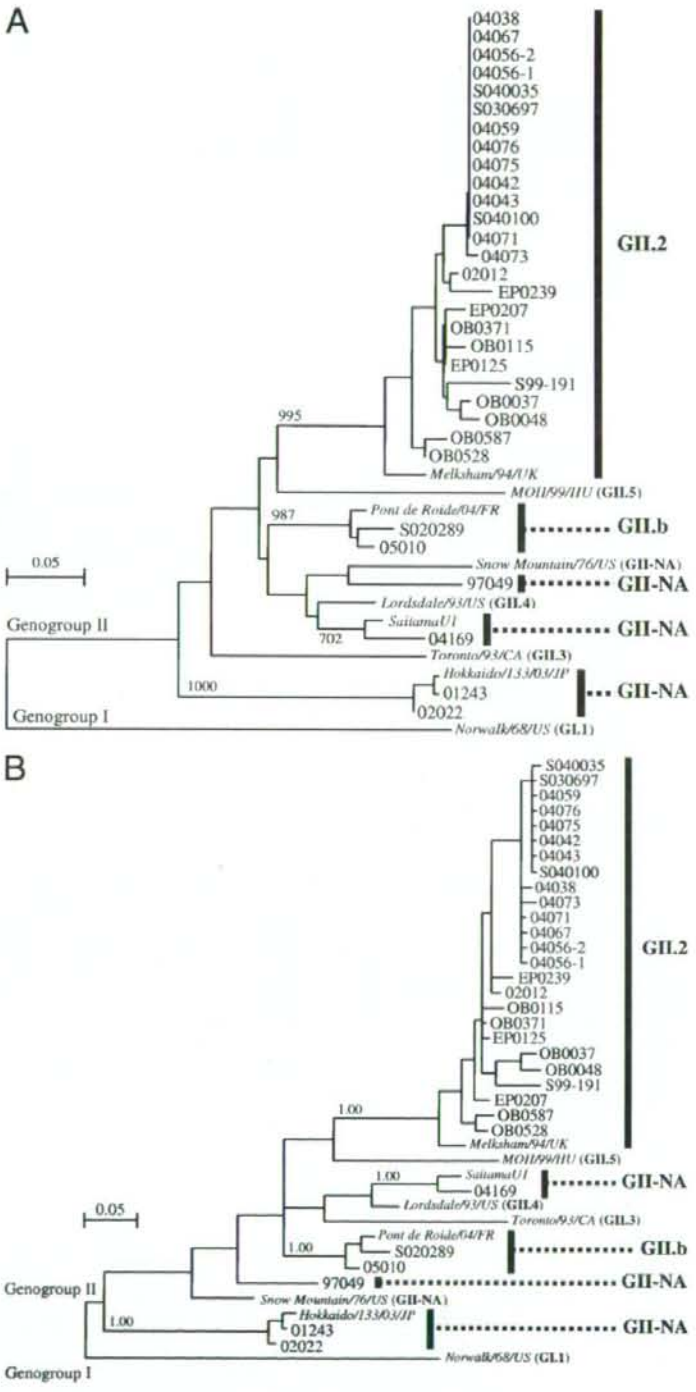

TABLE 2. Primers used to amplify the capsid gene of GII.2 NoV strains

Primer	Sequence (5′ to 3′)[a]	Polarity	Location (nt)	Reference or source
COG2F	CARGARBCNATGTTYAGRTGGATGAG	+	5003[b]	19
G2SKF	CNTGGGAGGGCGATCGCAA	+	5058[b]	22
G2SKR	CCRCCNGCATRHCCRTTRTACAT	−	5401[b]	22
N235Rex	GCWANRAAAGCTCCWGCCAT	−	6273[b]	17
MKcap508-524F	CAGAAAGATGATCCCAA	+	508[c]	This study
MKcap524-508R	TTGGGATCATCTTTCTG	−	524[c]	This study
MKcap662-649R	TCCACTGTTGGTGG	−	662[c]	This study
MKcap1109-1125F	TGGGTCAGATTCAAATT	+	1109[c]	This study
MKcap1125-1109R	AATTTGAATCTGACCCA	−	1125[c]	This study
MKcap1304-1290R	AAGAGCAGGCGCTCC	−	1304[c]	This study
MK35R	CAAAAGCTCCAGCCAT	−	1644[c]	This study

[a] Abbreviations for residues in boldface are as follows: B, not A; H, not G. In addition, N is A, C, G, or T; R is A or G; W is A or T; Y is C or T.
[b] Location of the 5′ end of the primer corresponding to the nucleotide position of Lordsdale/93/UK (X86557).
[c] Location of the 5′ end of the primer corresponding to the nucleotide position of ORF2 and ORF3 of Melksham/94/UK (X81879).

and EsyPred3D (25) servers. WHAT IF could not model residues 342, 344, and 345 based on the Norwalk/68/US capsid protein template (PDB ID 1IHM) as they are present in an inserted loop of the P2 subdomain relative to the template structure, but they are predicted to be in the same position as in the 3D model based on the GII.4 genotype capsid protein template (PDB ID 2OBR). A dimer of the GII.2 NoV capsid protein was modeled by superimposing two predicted monomers onto the trimeric template of the Norwalk/68/US capsid protein. The 3D models were visualized by the YASARA view program (version 6.813; http://www.yasara.org) (24).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank with the accession numbers AB281081 to AB281090 (Table 1).

RESULTS

Capsid gene sequence analysis of GII.2 strains collected over 30 years. Sequence data from a total of 36 GII.2 strains showed that the capsid gene was 1,629 nt long and coded for a protein of 542 amino acids. There were no deletions or insertions in the capsid gene among these strains collected over a 30-year period. Sequence comparison showed ≥83.6% nucleotide and ≥93.5% amino acid identities among these GII.2 strains. The phylogenetic tree based on the 1,629 nucleotide sequences of the complete capsid gene confirmed that all strains were characterized as GII.2 genotype (Fig. 2).

Genetic analysis of GII.2 strains from a regional outbreak in Japan in the spring of 2004. Of the 21 GII.2 strains detected in Osaka City, Japan, 14 strains were detected in samples from cases in outbreaks or sporadic cases in the spring epidemic between March and May 2004 (Table 1). These strains were closely related to each other (≥99.2% nucleotide and ≥ 99.1% amino acid identities). To find out if these viruses changed genetically during circulation in the community for a short period of time (about 3 months), the complete nucleotide and amino acid sequences of the capsid gene from these 14 GII.2 strains were compared. A total of 45 nucleotide substitutions were observed (Table 3). The majority were third-base position changes (77.8%) and synonymous substitutions (82.2%). These nucleotide changes resulted in amino acid changes in eight codons, five of which were located in the P2 subdomain. In the alignment of the spring epidemic strains over the 3-month period, of the 45 nucleotide substitutions, only one nucleotide position was fixed (nt 594 in the S domain) at the end of this epidemic. This did not result in an amino acid change. All amino acid changes were sporadic. Eight of the 14 strains had an identical amino acid sequences (Table 1). Of these, strain 04038 was used for further genetic analysis.

Genetic analysis of all GII.2 strains collected between 1976 and 2005. The nucleotide and amino acid sequences of the complete capsid genes were compared for 29 GII.2 strains collected between 1975 and 2005 (30 years), excluding 7 GII.2 strains detected in Osaka City with identical amino acid sequences (10 strains from The Netherlands, 14 from Osaka City, and 5 from GenBank). A total of 488 nucleotide changes were observed (Table 4). Again, the majority of these were third-base changes (83.8%) and synonymous (85.9%). In total, 59 nucleotide changes resulted in amino acid changes (34 in the P2 subdomain) (Table 4). Twenty-five of these were informative changes (19 in the P2 subdomain), but none appeared to be fixed in the genome over time. Of the 25 informative sites, the amino acid position 345 was the most variable (Fig. 3). Nevertheless, statistical analysis showed a significantly higher rate of mutation in the P2 subdomain than in P1 and S, suggesting selective pressure ($P = 0.0018$, chi-square 5.63; and $P < 0.0001$, chi-square 12.9).

Genetic analysis of Melksham-like strains detected between 1994 and 2005. In order to understand the apparent discrepancy between selective changes in the P2 subdomain and the absence of fixation of these mutations, we repeated our anal-

FIG. 1. Phylogenetic analysis of partial RdRp gene sequences of GII.2 NoV strains by the neighbor-joining method (A) and the Bayesian method (B). The genotypes at the RdRp regions that are not assigned numbers are represented as GII-NA. The scale indicates the number of substitutions per site. Reference strains of NoV used in this analysis are given in italics. (A) The bootstrap values are indicated on each branch. (B) Tree topology was evaluated on the basis of 1,500,000 generations. The posterior probabilities are indicated on each branch (≥0.95 of the posterior probability means that the branch has high credibility). The GenBank accession numbers of the additional reference strains used in this analysis are as follows: Hokkaido/133/03/JP, AB212306; Lordsdale/93/UK, X86557; MOH/99/HU, AF397256; Norwalk/68/US, M87661; Pont de Roide 673/04/FR, AY682549; Saitama U1, AB039775; Toronto/93/CA, U02030. For strain abbreviations, see Table 1.

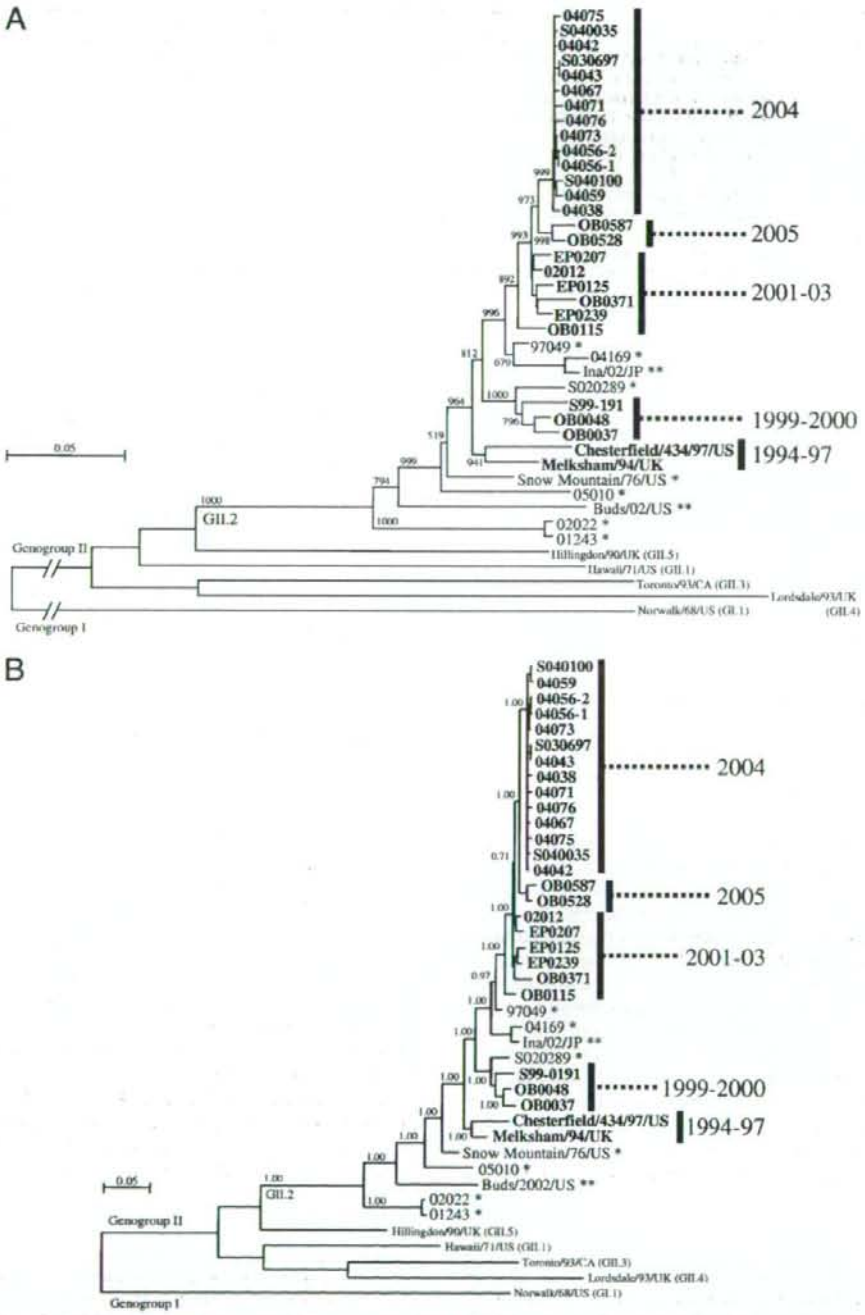

FIG. 2. Phylogenetic analysis of complete capsid gene sequences of GII.2 NoV strains by the neighbor-joining method (A) and the Bayesian method (B). Melksham-like strains, which have a matching (GII.2) RdRp sequence, are shown in boldface. Asterisks indicate the GII.2 strains that have different (*) or unknown (**) genotypes in the RdRp region. The scale indicates the number of substitutions per site. (A) The bootstrap values are indicated on each branch. (B) Tree topology was evaluated on the basis of 500,000 generations. The posterior probabilities are indicated on each branch. The GenBank accession numbers of the additional reference strains used in this analysis are as follows: Hawaii/71/US, U07611; Hillingdon/90/UK, AJ277607; Lordsdale/93/UK, X86557; Norwalk/68/US, M87661; Toronto/93/CA, U02030. For strain abbreviations, see Table 1.

TABLE 3. The numbers of nucleotide and amino acid substitutions in the complete capsid gene among 14 GII.2 strains detected in the spring epidemic of 2004 in Osaka City, Japan

Domain or subdomain[a]	Nucleotide substitutions						Amino acid substitutions	
	No. of substitutions	No. at the indicated position of the changed codon			Synonymous changes (no.)	Nonsynonymous changes (no.)	No. of substitutions	Informative changes (no.)
		1st	2nd	3rd				
N	1	0	0	1	1	0	0	0
S	18	3	2	13	16	2	2	0
P1	7	0	0	7	6	1	1	0
P1-1	4	0	0	4	4	0	0	0
P1-2	3	0	0	3	2	1	1	0
P2	19	3	2	14	14	5	5	0
Total	45	6	4	35	37	8	8	0

[a] Each domain of GII.2 NoV capsid gene was determined according to Chen et al. (7). The region and abbreviation of each domain are as follows: N, 5′ end of ORF2 and N terminal domain (residues 1 to 45); S, shell domain (residues 46 to 216); P1, P1 subdomain (P1-1, residues 217 to 274; P1-2, residues 421 to 542); P2, P2 subdomain (residues 275 to 420).

ysis after removing recombinant genomes from the alignment (Table 5) (nucleotide and amino acid sequences of the complete capsid gene from 20 Melksham-like strains: 10 strains from The Netherlands, 8 from Osaka City, and 2 from GenBank). Sequence comparison showed ≥91.5% nucleotide and ≥97.4% amino acid identities among these Melksham-like strains. A total of 301 nucleotide changes were observed (Table 5), the majority of which were third-base changes (87%) and synonymous (88.7%). These nucleotide changes resulted in amino acid changes in 32 codons, half of which were located in the P2 subdomain. Twelve of 32 amino acid positions were informative (9 in the P2 subdomain) (Fig. 3). In contrast with the previous finding, several mutations were fixed: of the 12 informative sites, 2 amino acid substitutions (amino acid positions 245 and 440) in the P1 subdomain and 3 amino acid substitutions (amino acid positions 342, 344, and 345) in P2 subdomain were cumulative (Table 6), segregating the strains into five genetic groups (1994 to 1997, 1999 to 2000, 2001 to 2003, 2004, and 2005) by the neighbor-joining method (Fig. 2A) and Bayesian method (Fig. 2B).The strains detected in the spring epidemic in Osaka City had a unique sequence, with S or P residues at amino acid position 364 (Fig. 3 and Table 6). The other six informative sites were not fixed.

The 3D structure of the P domain of a monomer of the NoV capsid protein was predicted by WHAT IF, 3D-Jigsaw, and EsyPred3D, based on the known 3D structure of the VA387/98/US GII.4 genotype capsid protein, which has 55% amino acid sequence identity in the P domain to the Melksham capsid protein. A comparison of the positions of the six fixed mutations to the predicted 3D structure indicated that all six residues were predicted to be located at the surface of the capsid protein, with three residues (342, 344, and 345) close to each

TABLE 4. The numbers of nucleotide and amino acid substitutions in the complete capsid gene among 29 GII.2 strains collected in the GenBank, The Netherlands, and Japan over a 30-year period

Domain or subdomain[a]	Nucleotide substitutions						Amino acid substitutions	
	No. of substitutions	No. at the indicated position of the changed codon			Synonymous changes (no.)	Nonsynonymous changes (no.)	No. of substitutions	Informative changes (no.)
		1st	2nd	3rd				
N	20	0	2	18	17	3	3	1
S	137	9	3	125	131	6	6	1
P1	162	17	7	138	143	19	16	4
P1-1	53	4	2	47	49	4	3	2
P1-2	109	13	5	91	94	15	13	2
P2	169	28	13	128	128	41	34	19
Total	488	54	25	409	419	69	59	25

[a] See note to Table 3.

FIG. 3. Amino acid alignment of the P2 subdomains of GII.2 NoV strains showing mutations along the aligned sequences. The upper sequence alignment group (A) includes the Melksham-like strains and the lower group (B)includes the other GII.2 strains, which were recombinant genomes. In each group, sequences are arranged chronologically from top (oldest) to bottom (most recent). The detection years of the strains are indicated in parentheses. The asterisk indicates informative sites among Melksham-like strains. The arrow denotes cumulatively changing amino acid positions (342, 344, and 345) among Melksham-like strains. The numbers above the sequences indicate the sequence position relative to the position in the capsid protein of strain Melksham. Underlined sequences indicate the KGE motif that corresponds to the RGD-like motif of other NoVs and was determined with the amino acid sequence alignment of other NoVs according to Tan et al. (34). For strain abbreviations, see Table 1.

other in the P2 subdomain (Fig. 4). Furthermore, residues 342, 344, 345 of the P2 subdomain and residues 245 and 440 of the P1 subdomain were grouped closely together on the predicted 3D structure of a dimer (3D-modeling by WHAT IF) (Fig. 5).

DISCUSSION

In this study, we analyzed the complete capsid gene of GII.2 NoVs collected over a 30-year period. The collection included a relatively large sample from an epidemic that was observed only in Japan in the spring period of 2004. In this 3-month period, no evolutionary changes were observed, but compared with other GII.2 Melksham-like strains these variants had unique amino acid sequences (S or P) at position 364. One of the recombinant genomes, strain 02022, had the same amino acid residues, suggesting that the presence of this mutation was not causally related to the epidemic pattern. The sequence analysis confirmed that the spring epidemic was an outbreak.

Molecular characterization of the GII.2 capsids over the full study period showed an interesting difference between recombinant genomes and nonrecombinant (Melksham-like) genomes. The Melksham-like strains clearly evolved over time, with accumulation of mutations particularly in the P2 subdomain. In contrast, GII.2 capsids from recombinant genomes did not fit this pattern and had a seemingly erratic pattern of mutations. Melksham-like strains are occasionally observed in molecular surveillance data from The Netherlands and Osaka

TABLE 5. The numbers of nucleotide and amino acid substitutions in the complete capsid gene among 20 Melksham-like strains over a 12-year period

Domain or subdomain[a]	Nucleotide substitutions						Amino acid substitutions	
	No. of substitutions	No. at the indicated position of the changed codon			Synonymous changes (no.)	Nonsynonymous changes (no.)	No. of substitutions	Informative changes (no.)
		1st	2nd	3rd				
N	10	0	2	8	8	2	2	0
S	81	4	3	74	76	5	6	1
P1	98	8	3	87	89	9	8	2
P1-1	34	2	1	31	32	2	2	1
P1-2	64	6	2	56	57	7	6	1
P2	112	13	6	93	94	18	16	9
Total	301	25	14	262	267	34	32	12

[a] See note to Table 3.

TABLE 6. Amino acid substitutions at six positions in the P domain among Melksham-like strains

Strain description			Amino acid and codon at the indicated position of the capsid protein[a]											
			245		342		344		345		364		440	
Name[b]	Country[c]	Detection date (mo-day-yr)	Residue	Codon	Residue	Codon	Residue	Codon	Residue	Codon	Residue	Codon	Residue	Codon
Melksham	UK	1994	P	CCC	Q	CAG	A	GCC	A	GCT	A	GCA	L	CTC
CF434	US	1997	—	CCC	—	CAG	—	GCT	—	GCT	—	GCA	—	CTT
S99-191	NL	1-1999	S	TCT	—	CAA	—	GCC	T	ACT	—	GCA	I	ATC
OB0037	NL	9-2000	S	TCT	—	CAA	—	GCC	T	ACT	—	GCA	I	ATC
OB0048	NL	12-2000	S	TCT	—	CAA	—	GCC	T	ACT	—	GCA	I	ATC
OB0115	NL	2-2001	S	TCT	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
EP0125	NL	11-2001	S	TCC	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
02012	JP	1-2002	S	TCC	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
EP0207	NL	1-2002	S	TCC	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
EP0239	NL	2-2002	S	TCC	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
OB0371	NL	10-2003	S	TCC	H	CAC	—	GCC	S	TCT	—	GCA	I	ATC
04038	JP	3-11-2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
04059	JP	4-18–30-2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
S040035	JP	4-21-2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
04071	JP	5-16–23-2004	S	TCC	H	CAC	S	TCC	P	CCT	P	CCA	I	ATC
04073	JP	5-22-2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
04075	JP	5-25-2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
S040100	JP	5-31-2004	S	TCC	H	CAC	S	TCC	P	CCT	S	TCA	I	ATC
OB0528	NL	1-2005	S	TCC	H	CAC	S	AGC	P	CCT	—	GCA	I	ATT
OB0587	NL	4-2005	S	TCC	H	CAC	S	AGC	P	CCT	—	GCA	I	ATT

[a] The position number corresponds to the capsid sequence of the Melksham/94/UK strain; a dash indicates sequence identity with this strain. Residues are indicated by their single-letter codes.

[b] For strain abbreviations, see Table 1.

[c] UK, United Kingdom; US, United States; NL, The Netherlands; JP, Japan.

City, and strains from Japan and The Netherlands could be consistently grouped in the same alignment, suggesting continuous widespread circulation and an ability to cause disease. The recombinant genomes with GII.2 capsids, however, occur sporadically and did not show evidence for evolution, suggesting that their circulation is limited (1, 4, 17). The finding that strains from such widely separated geographic regions were similar shows that evolution of GII.2 NoVs is a global phe-

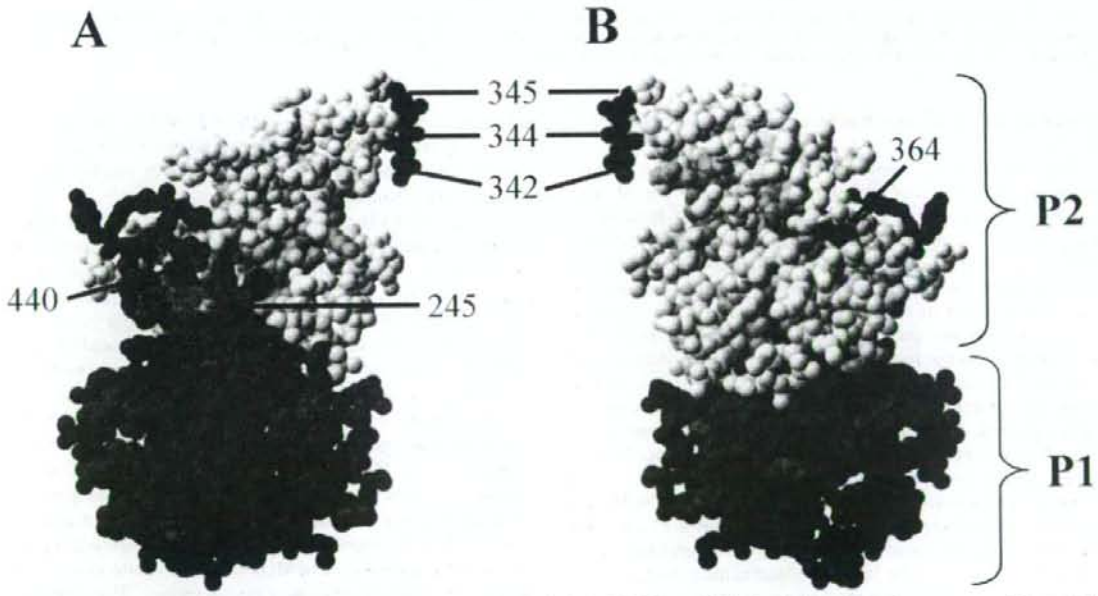

FIG. 4. Location of six fixed amino acid residues (positions 245, 342, 344, 345, 364, and 440, shown in red) on the monomer of the capsid protein. This 3D structure for the monomer P domain of the GII.2 NoV capsid protein was made by WHAT IF. The P1 and P2 subdomains are shown in blue and gray, respectively. The S domain is not shown. The KGE motif in the P2 domain is shown in green. (A) Predicted location of amino acid residues 342, 344, 345, and 364 in the P2 subdomain. (B) A view identical to panel A rotated 180° horizontally showing the location of amino acid residues 245 and 440 in the P1 subdomain.

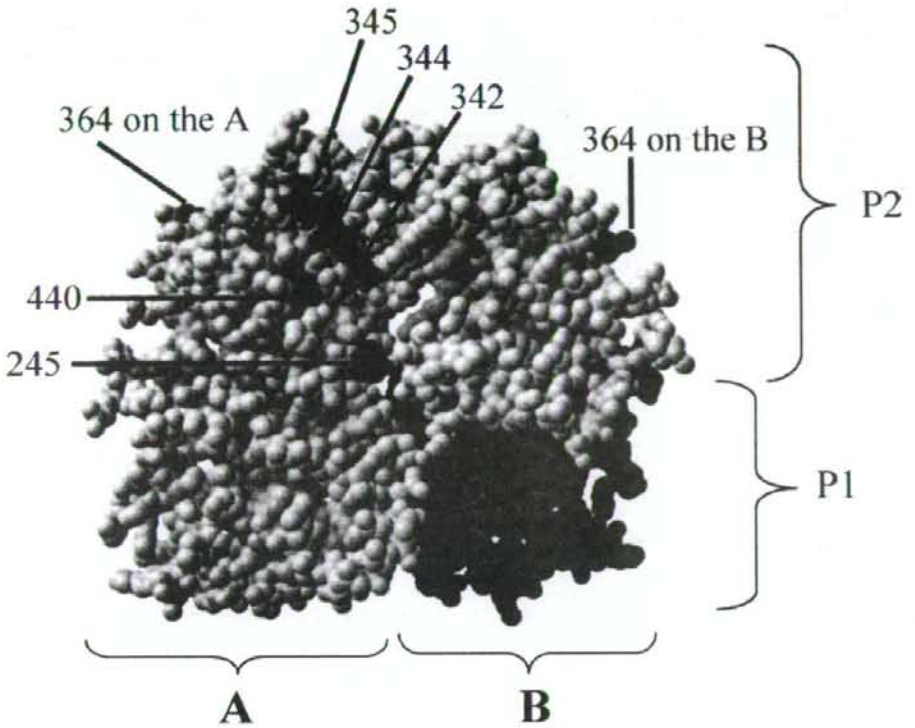

FIG. 5. The 3D structure of a dimer of the GII.2 NoV capsid protein, as predicted by WHAT IF, showing the location of the six informative amino acid residues (red). Monomer A is shown in yellow, while the monomer B is shown in blue (P1) and gray (P2). The S domains are not shown. The KGE motif (green) in the P2 subdomain on monomer B is also shown.

nomenon, similar to what has been described for GII.4 NoVs (5, 27, 33).

The molecular characterization of Melksham-like strains showed accumulation of mutations over a 12-year period, segregating them into five genetic groups. Three of these were supported in phylogenetic analysis with high bootstrap values. Two others (Fig. 2, 1999 to 2000 and 2001 to 2003) were not distinguishable in phylogenetic analysis, possibly because of the small number of isolates in the comparison. The present collection, however, contains all GII.2 strains detected in the two countries in the past 12 years of surveillance. Amino acid position 345 was the most variable in all GII.2 strains. This is suggestive of immune pressure, particularly because the amino acid is predicted to be located on a surface-exposed part of the capsid. The fixed amino acid changes were topologically clustered, judging from the 3D structure prediction, indicating that combined they may form an epitope. Immunity to NoVs has not been studied very systematically, but recent work on GII.4 NoVs has suggested that these viruses evolved under immune pressure (9, 30, 33). The lack of a tissue culture model makes it difficult to corroborate these findings by using cross-neutralization tests with hyperimmune serum directed against specific variants. Evolution of strains in an immunocompromised patient with chronic shedding of NoVs has been demonstrated by Nilsson et al. (28), who suggested that the cumulative amino acid substitutions appearing in the P2 subdomain were immune response driven. To clarify the relations between these amino acid mutations and receptor or antibody binding further immunological studies are needed.

Recently, new variants of the predominant NoV, the GII.4 genotype, appeared in 2002 (27), 2004 to 2005 (5), and 2006 (33) with global distribution. These new GII.4 variants were characterized into different clusters by the phylogenetic analysis based on the capsid gene (5, 9, 30). Okada et al. (30) reported that GII.4 strains had cumulative amino acid changes in the P domain and 5% to 18% amino acid diversity in the P2 subdomain between GII.4 subtypes for 5 years study. Bull et al. (5) also described more than 5% amino acid diversity in the complete capsid gene between past GII.4 strains and variants. Siebenga et al. (33) found 9% fixed amino acid mutations across the capsid gene over a 12-year period, but only five of these consistently delineated subsequent epidemic strains. In contrast, here we found only 2.6% amino acid diversity across the complete capsid gene of Melksham-like strains in a 12-year period, showing a clearly lower rate of change. The difference between these genotypes is intriguing and suggests clear differences in epidemiology that are not easy to explain. This study has been done using a limited number of strains in a rare genotype from a limited number of locations. It is necessary to

continue molecular surveillance of NoV infections to further the evolutional analysis of each genotype.

In conclusion, the present study showed that NoVs belonging to a rare genotype evolved by the accumulation of mutations in the surface-exposed parts of the capsid, suggesting immune response-driven evolution. The pattern of change similar to what has been observed for GII.4 is a global one, suggesting that these viruses circulate much more than is apparent from their presence in surveillance data. The data also showed for the first time that recombinant genomes of NoVs behave differently, even when they share the same capsid genes as nonrecombinant genomes. Understanding how NoVs evolve is necessary for finding more effective ways to control this disease and particularly its impact in healthcare settings.

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Norovirus and Sapovirus Infections among Children with Acute Gastroenteritis in Ho Chi Minh City during 2005–2006

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Summary

A molecular epidemiological study on common diarrheal viruses was conducted in a children's hospital in Ho Chi Minh City between December 2005 and November 2006. Fecal samples were collected from 502 pediatric patients with acute gastroenteritis, and were screened for the presence of norovirus (NoV) and sapovirus (SaV). NoVs GII and SaVs were detected in 6.4% and 1.2% specimens, respectively, while there was no NoV GI found among studied samples. NoVs could be identified through the year, except in April and July, with the peak of detection rate (62.5%) during the rainy season. Conversely, four out of six (66.7%) of the SaV strains were identified during the dry season. Patients aged between 6 and 23 months were found to be more infected by NoVs. The overall mean severity score of norovirus-positive patients was 9.8 ± 3.6, and no significant difference of severity scores among patients belonged to different age groups, gender and place of living. The results of phylogenetic analysis showed the diversity of caliciviruses circulating in the area, and various types of recombination were identified among NoVs and SaVs detected. These results provide important information on calicivirus infections among Vietnamese children.

Key words: norovirus, sapovirus, clinical manifestations, recombinant, Vietnam.

Introduction

Norovirus (NoV) and sapovirus (SaV) are members of the family *Caliciviridae* (other two genera are *Lagovirus* and *Vesivirus*). The NoV and SaV strains are determined as the major causes of non-bacterial acute gastroenteritis in infants and young children [1, 2].

After being discovered through electron microscope in 1972 [3], NoVs were identified widely in epidemiological studies, and were the cause of outbreaks of gastroenteritis in various settings including hospitals [4, 5], schools [6, 7], cruise ships [8, 9], restaurants [10, 11] and day care centers [12, 13]. Sequence analyses of worldwide NoVs revealed that they are classified into seven distinct genogroups (GI to GVII), of these, GI, GII, GIV, GVI and GVII are known to infect humans [14]. NoV contains a positive-sense single-stranded RNA genome surrounded by an icosahedral capsid. The NoV genome contains three open reading frames (ORFs). The ORF1 encodes non-structural proteins, including NTPase, protease and RNA-dependent RNA polymerase (RdRp), OR2 encodes the capsid protein (VP1) and ORF3 encodes a minor structural protein (VP2).

SaV infects both children and adults, and have been found to cause outbreaks of gastroenteritis in kindergarten [15], hospital [16] and mental health care facility [17]. SaV- associated diarrhea is usually mild, compared to that caused by NoVs [18].

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