Human Sapovirus in Clams, Japan

Grant S. Hansman,* Tomoichiro Oka,* Reiko Okamoto,† Tomoko Nishida,† Shoichi Toda,† Mamoru Noda,‡ Daisuke Sano,§ You Ueki,¶ Takahiro Imai,§ Tatsuo Omura,§ Osamu Nishio,* Hirokazu Kimura,* and Naokazu Takeda*

Human sapovirus was detected in 4 of 57 clam packages by reverse transcription–PCR and sequence analysis. This represents the first finding of sapovirus contamination in food. Closely matching sequences have been detected in stool specimens from patients with gastroenteritis in Japan, which indicates a possible food-to-human transmission link.

Sapoviruses and noroviruses are etiologic agents of human gastroenteritis. Human noroviruses are the most important cause of outbreaks of gastroenteritis worldwide and can be transmitted by a variety of routes, including food (*1*). Sapovirus infections are mostly associated with sporadic gastroenteritis in young children; however, foodborne transmission routes are yet to be determined. The most widely used method of detection is reverse transcription–PCR (RT-PCR), which has a high sensitivity and can also be used for genetic analysis. Sapovirus strains can be divided into 5 genogroups; GI–GV infect humans; sapovirus GIII infects porcine species. Phylogenetic studies have also designated sapovirus clusters or genotypes to further describe strains.

The Study

The purpose of this study was to detect sapovirus in the clam *Corbicula japonica* (bivalve mollusk) and describe the genetic diversity of the strains. A total of 57 clam packages (30–60 clams per package) were collected from supermarkets or fish markets from 6 different areas in western Japan from December 8, 2005, to September 6, 2006. The samples were shucked, and the digestive diverticulum was removed by dissection and weighed. One gram of digestive diverticulum (10–15 clam/package) was homogenized with an Omini-mixer (Sorvall Inc., Newtown, CT, USA) in 10 mL phosphate-buffered saline. After centrifugation at 10,000× *g* for 30 min at 4°C, the supernatant was centrifuged at 100,000× *g* for 2 h (SW41 Rotor, Beckman Instruments, Inc., Fullerton, CA, USA). The pellet was resuspended in 140 μL distilled water and stored at –80°C until use.

RNA extraction and nested RT-PCR were performed as described (*2*). Briefly, for the first PCR, F13, F14, R13, and R14 primers were used; for the nested PCR, F22 and R2 primers were used. All RT-PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. RT-PCR products were excised from the gel and purified by the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). Nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1, Applied Biosystems, Warrington, England) and determined with the ABI 3130 Avant sequencer (ABI, Boston, MA, USA). Nucleotide sequences were aligned with ClustalX, and the distances were calculated by Kimura's 2-parameter method, as described elsewhere (*2*). Nucleotide sequence data determined in this study have been deposited in GenBank under accession nos. EF104251–EF104254.

Four (7%) of 57 clam packages were contaminated with sapovirus (termed Shijimi1, Shijimi2, Shijimi3, and Shijimi4). Genetic analysis of the partial capsid gene showed that these 4 sequences shared >98% nucleotide similarity and >97% amino acid identity. Phylogenetic analysis grouped these 4 sequences in the same genotype, i.e., GI/1 (Figure). Similar sequences were found on the database (Figure). Strains from this cluster likely represent the dominant genotype worldwide (*3*). Three of 4 sapovirus-positive clam packages were collected from different areas and at different times (Figure). The clam packages that were contaminated with Shijimi1 and Shijimi3 were collected from the same area, but 6 weeks apart, which indicates an ongoing sapovirus contamination or resistance in the natural environment. The seasonality of sapovirus infection in Japan is unknown; however, as with norovirus, sapovirus infections may also peak during winter, although further epidemiologic and environmental studies are needed.

In a recent study, we detected sapovirus strains in 7 of 69 water samples, which included untreated wastewater, treated wastewater, and a river in Japan (*4*). Three of 7 sapovirus sequences detected in the water samples belonged to GI/1 and shared >97% nucleotide similarity with the sapovirus sequences detected in the clam packages. Additionally, sapovirus sequences belonging to GI/1 and sharing >99% nucleotide similarity, for example, Chiba/010598F strain (Figure), have been detected in stool specimens from children with sporadic gastroenteritis in Japan (*5,6*). The closely matching sapovirus sequences detected in the water, clams, and patients suggest that

*National Institute of Infectious Diseases, Tokyo, Japan; †Yamaguchi Prefectural Research Institute of Public Health, Yamaguchi, Japan; ‡Hiroshima City Institute of Public Health, Hiroshima, Japan; §Tohoku University, Sendai, Japan; and ¶Miyagi Prefectural Institute of Public Health and Environment, Sendai, Japan

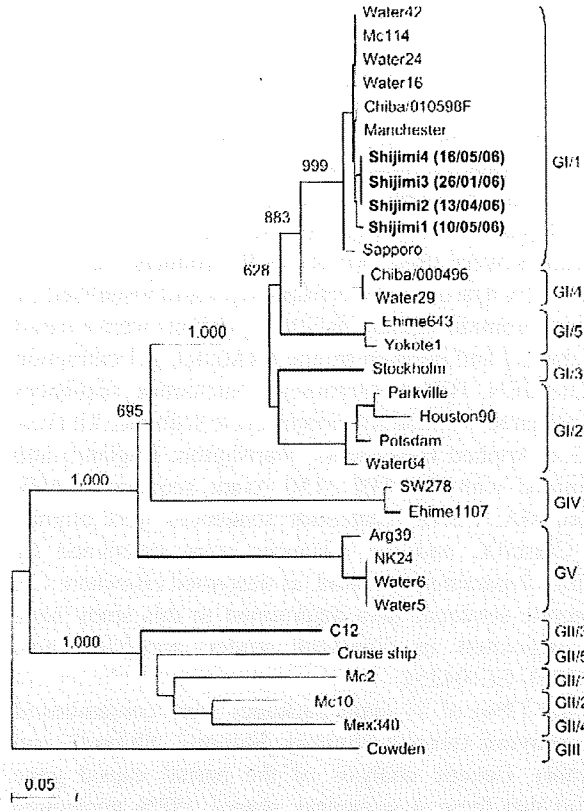

Figure. Phylogenetic analysis of sapovirus capsid sequences (≈300 nt) showing the different genogroups and clusters. Numbers on each branch indicate bootstrap values for the genotype. Bootstrap values of ≥950 were considered statistically significant for the grouping. The scale represents nucleotide substitutions per site. GenBank accession nos.: Mex340, AF435812; Parkville, U73124; Cowden, AF182760; Potsdam, AF294739; Sapporo, U65427; Stockholm, AF194182; SW278, DQ125333; water samples, DQ915088–DQ915094; and Yokote, AB253740. **Boldface** represents sequences detected in this study.

sapovirus contamination in the natural environment can lead to foodborne infections in humans, although direct evidence is lacking. More important, a recent study found animal sapovirus in oysters and suggested that coinfection with human and animal sapovirus strains could result in genomic recombination and the emergence of new strains (*7*). At the same time, we recently described the first human sapovirus intergenogroup recombinant strain (*8*). Phylogenetic analysis of the nonstructural region (i.e., genome start to capsid start) grouped this sapovirus strain in GII, while the structural region (i.e., capsid start to genome end) grouped this strain in GIV.

A large number of studies have detected norovirus in oysters. In 2 recent studies, norovirus was detected in oysters (*Crassosterea gigas*) harvested from geographically isolated areas in Japan (*9,10*). We also screened the same oyster samples for sapovirus; however, all of the samples were negative for sapovirus. That sapovirus was detected in the clam samples, but not in the oyster samples, is of interest. In the past several years, increasing evidence has emerged that human noroviruses bind to histo-blood group antigens (HBGAs) (*11*). These carbohydrate epitopes are present in mucosal secretions and throughout many tissues of the human body, including the small intestine, and in oyster digestive tissues. A number of studies have found that different norovirus strains exhibit different binding patterns to HBGAs and oyster digestive tissues (*12,13*). In a recent study, we found that sapovirus GI and GV strains showed no such binding activity to HBGAs (*14*). These results suggest that human norovirus and sapovirus strains have different binding receptors or that human sapovirus may not concentrate in detectable levels in oysters.

Conclusions

Foodborne diseases are a major problem worldwide. We report what is, to the best of our knowledge, the first account of sapovirus contamination in food destined for human consumption. The report may represent a possible food-to-human transmission link, although direct evidence is lacking. In Japan, clams are usually boiled before they are consumed in soups. However, boiling to open the clam may not inactivate the virus (*15*); in addition, some areas in Japan do not boil clams before eating them. Further studies are needed to determine if boiling inactivates sapovirus and if the contaminated clams are indeed infectious. In conclusion, these novel results highlight the importance of sapovirus, in particular the GI/1 strains. A new awareness of sapovirus transmission routes is necessary and may help reduce sapovirus infections.

Dr Hansman is a scientist at the National Institute of Infectious Diseases, Japan. He studies viruses that cause gastroenteritis in humans, namely sapovirus and norovirus. His research interests include epidemiology, virus expression, and cross-reactivity.

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Address for correspondence: Grant S. Hansman, Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo, 208-0011, Japan; email: ghansman@nih.go.jp

Recombinant Sapovirus Gastroenteritis, Japan

To the Editor: Sapovirus and norovirus are causative agents of gastroenteritis in children and adults. Norovirus is the most important cause of outbreaks of gastroenteritis, whereas only a few outbreaks of sapovirus have been reported (*1*,*2*). On the basis of complete capsid gene sequences, sapovirus can be divided into 5 genogroups, among which GI, GII, GIV, and GV infect humans, whereas sapovirus GIII infects porcine species.

We report 2 outbreaks of gastroenteritis in Hokkaido, Japan. The first outbreak (A) occurred at a college from May 29 to June 2, 2000. A total of 12 persons (11 students and 1 teacher) reported symptoms of gastroenteritis (nausea, vomiting, stomachache, diarrhea, and fever); 11 stool specimens were collected from days 1 to 7 after onset of illness (Table). These specimens were negative for norovirus (data not shown), but 5 were positive for sapoviruslike viruses by electron microscopy (Table).

The 11 specimens were then examined for sapovirus by using nested reverse transcription–PCR (RT-PCR) as described (*3*). A total of 9 (82%) of 11 specimens were positive for sapovirus. Sequence analysis showed that these 9 viruses had 100% nucleotide identity and likely represented the same sapovirus strain (termed Yak2 strain, GenBank accession no. AB046353). To determine the number of cDNA copies per gram of stool, we performed real-time RT-PCR as described (*4*). The number of sapovirus cDNA copies ranged from 5.36×10^5 to 7.47×10^9/g stool (median 5.49×10^9 copies/g stool) (Table).

The second outbreak (B) occurred at a kindergarten from February 1 to 22, 2005. A total of 23 persons (15 children and 8 adults) reported symp-

Table. Analysis of 18 stool specimens for sapovirus during 2 outbreaks of gastroenteritis, Japan*

Outbreak, specimen	Date of illness onset	EM	Nested RT-PCR	Real-time PCR†
A				
Yak1	Jun 2, 2000	–	+	7.47×10^9
Yak2	May 29, 2000	–	–	–
Yak3	May 30, 2000	–	–	–
Yak4	May 31, 2000	+	+	6.55×10^9
Yak5	Jun 1, 2000	–	+	9.38×10^8
Yak6	Jun 1, 2000	–	+	1.30×10^8
Yak7	May 29, 2000	–	+	1.46×10^9
Yak8	May 29, 2000	+	+	2.78×10^{10}
Yak9	Jun 1, 2000	+	+	3.00×10^9
Yak10	Jun 1, 2000	+	+	2.05×10^9
Yak11	Jun 1, 2000	+	+	5.36×10^5
B				
Nay1	Feb 17, 2005	NT	+	1.65×10^{10}
Nay2	Feb 14, 2005	NT	+	1.82×10^9
Nay3	Feb 18, 2005	NT	+	1.14×10^9
Nay4	Feb 17, 2005	NT	+	5.41×10^{10}
Nay5	Feb 16, 2005	NT	+	5.26×10^{10}
Nay6	Feb 18, 2005	NT	+	2.50×10^{10}
Nay7	Feb 17, 2005	NT	+	2.38×10^{10}

*EM, electron microscopy; RT-PCR, reverse transcription–PCR; NT, not tested.
†cDNA copies/g stool.

toms of gastroenteritis (nausea, vomiting, stomachache, diarrhea, and fever); 7 stool specimens were collected (Table). These specimens were negative for norovirus (data not shown), but all were positive for sapovirus by nested RT-PCR. The 7 sequences from this outbreak had 100% nucleotide identity and likely represented the same sapovirus strain (termed Nay1 strain, GenBank accession no. EF213768). The number of sapovirus cDNA copies ranged from 1.14×10^9 to 5.41×10^{10}/g stool (median 2.50×10^{10} copies/g stool) (Table).

One positive sapovirus specimen from each outbreak was subjected to further sequence analysis in which a single overlapping PCR fragment covering the partial polymerase gene and capsid gene was amplified. The Yak2 and Nay1 sequences shared ≈71% nucleotide identity for this fragment and likely represented different sapovirus strains. The Yak2 sequence closely matched sapovirus GIV Ehime1107 and SW278 sequences (GenBank accession nos. DQ058829 and AY237420, respectively) and had 98% and 97% nucleotide identity for the entire fragment, respectively (*5*). The Nay1 sequence closely matched the sapovirus GII C12 sequence (AY603425) and had 91% nucleotide identity for the entire fragment.

The Nay1 sequence closely matched the C12 sequence, which was detected in Osaka, Japan, in 2001 (*6*), whereas the Yak2 sequence closely matched the Ehime1107 sequence, which was detected in Matsuyama, Japan, in 2002 (*5*), and the SW278 sequence, which was detected in Sweden in 2003 (*1*). We recently described the C12 strain as intragenogroup recombinant sapovirus strain (*6*), whereas the Ehime1107 and SW278 strains were described as intergenogroup recombinant sapovirus strains (*5*). Our results indicate that recombination sites in intragenogroup and intergenogroup recombinant sapovirus strains were at the polymerase and capsid junction (*5,6*). Sapovirus Sydney53 (DQ104360) and Sydney3 strains (DQ104357), which were detected in Australia from August 2001 to August 2004 (*7*), closely matched C12 and Ehime1107/SW278 sequences, respectively. These results showed that recombinant sapovirus strains are stable in the environment and may be globally distributed. Our findings also suggest a changing distribution of sapovirus-associated gastroenteritis in Hokkaido because different sapovirus GI strains were predominant in outbreaks of gastroenteritis in Hokkaido (*8,9*).

In a recent study, the number of norovirus cDNA copies per gram of stool specimen was analyzed and a discrepancy was found between the different norovirus genogroups (*10*). Chan et al. found that noroviruses GI and GII showed medians of 8.4×10^5 and 3.0×10^8 copies/g of stool specimen, respectively, and speculated that increased viral loads were caused by higher transmissibility of norovirus GII strains (*10*). Our results showed that sapovirus GII Nay1 and GIV Yak2 strains showed higher viral loads than norovirus GII strains. These results suggest that a high degree of shedding of sapovirus GII Nay1 and GIV Yak2 strains may have caused the outbreak of gastroenteritis. However, to elucidate this suggestion, further studies are needed with other sapovirus strains.

This study was supported in part by a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan, and a grant for Research on Health Science Focusing on Drug Innovation from The Japan Health Science Foundation.

Grant S. Hansman,* Setsuko Ishida,† Shima Yoshizumi,† Masahiro Miyoshi,† Tetsuya Ikeda,† Tomoichiro Oka,* and Naokazu Takeda*

*National Institute of Infectious Diseases, Tokyo, Japan; and †Hokkaido Institute of Public Health, Hokkaido, Japan

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Address for correspondence: Grant S. Hansman, Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan; email: ghansman@nih.go.jp

JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2007, p. 3996–4005 Vol. 45, No. 12
0095-1137/07/$08.00+0 doi:10.1128/JCM.01516-07

Norovirus Infections in Symptomatic and Asymptomatic Food Handlers in Japan▽

Kazuhiro Ozawa,[1] Tomoichiro Oka,[2] Naokazu Takeda,[2] and Grant S. Hansman[2]*

Chubu Food and Environmental Safety Center, Shizuoka,[1] and Department of Virology II, National Institute of Infectious Diseases, Tokyo,[2] Japan

Received 29 July 2007/Returned for modification 11 August 2007/Accepted 29 September 2007

Noroviruses are the leading cause of outbreaks of gastroenteritis in the world. At present, norovirus genogroup II, genotype 4 (GII/4), strains are the most prevalent in many countries. In this study we investigated 55 outbreaks and 35 sporadic cases of norovirus-associated gastroenteritis in food handlers in food-catering settings between 10 November 2005 and 9 December 2006 in Japan. Stool specimens were collected from both symptomatic and asymptomatic individuals and were examined for norovirus by real-time reverse transcription-PCR; the results were then confirmed by sequence analysis. Norovirus was detected in 449 of 2,376 (19%) specimens. Four genogroup I (GI) genotypes and 12 GII genotypes, including one new GII genotype, were detected. The GII/4 sequences were predominant, accounting for 19 of 55 (35%) outbreaks and 16 of 35 (46%) sporadic cases. Our results also showed that a large number of asymptomatic food handlers were infected with norovirus GII/4 strains. Norovirus GII had a slightly higher mean viral load (1 log unit higher) than norovirus GI, i.e., 3.81×10^8 versus 2.79×10^7 copies/g of stool. Among norovirus GI strains, GI/4 had the highest mean viral load, whereas among GII strains, GII/4 had the highest mean viral load (2.02×10^8 and 7.96×10^9 copies/g of stool, respectively). Importantly, we found that asymptomatic individuals had mean viral loads similar to those of symptomatic individuals, which may account for the increased number of infections and the predominance of an asymptomatic transmission route.

The positive-sense polyadenylated single-stranded RNA virus family *Caliciviridae* contains four genera: *Norovirus*, *Sapovirus*, *Lagovirus*, and *Vesivirus* (1). The prototype strain of human norovirus is the Norwalk virus (NV/Human/US/1968), which was first discovered in an outbreak of gastroenteritis in an elementary school in Norwalk, OH, in 1968 (15). Noroviruses are the leading cause of outbreaks of gastroenteritis in the world; they cause outbreaks in various settings, including hospitals, cruise ships, schools, and restaurants (2, 9, 12, 15, 23, 24, 29). In addition, noroviruses have been detected in environmental samples (e.g., treated and untreated sewage) as well as in contaminated foods such as oysters, shellfish, sandwiches, salads, raspberries, and even ice (7, 18, 19, 26). Numerous molecular epidemiological studies have revealed a global distribution of these viruses (25, 27, 31).

The most widely used method of detecting noroviruses is reverse transcription-PCR (RT-PCR), which has high sensitivity; also, the products can be used for further genetic analysis. Real-time RT-PCR assays have also been developed; they are sensitive, broadly reactive, and rapid for the detection of human noroviruses in clinical stool specimens and environmental samples (13, 14, 21).

As the detection methods become more and more sensitive, the numbers of genogroups and genotypes are expected to increase. One emerging characteristic is that strains have been found to persist in one geographical region, only to disappear suddenly (8, 10). Seasonal studies have commonly found norovirus outbreaks peaking in the winter periods; however, the incidence rates, detection rates, and overall prevalence rates of infections may differ by country and setting and are likely to be affected by diagnostic techniques.

Recently, noroviruses have been divided into five genetically distinct genogroups, but the majority of human noroviruses can be divided into two genetically distinct genogroups, genogroup I (GI) and GII, which can be subdivided into at least 14 GI and 17 GII genotypes (14). Norovirus genotype identities are generally maintained across the open reading frames (ORFs). However, a number of norovirus strains failed to maintain their sequence identities for RNA-dependent RNA polymerase and VP1, and they were shown to be recombinant (16, 20, 30). Evidence suggested that the recombination site occurred at the conserved polymerase and capsid junction between ORF1 and ORF2.

The purpose of this study was to investigate norovirus-associated gastroenteritis in food handlers at food-catering settings in Japan between 10 November 2005 and 9 December 2006. Four GI genotypes and 12 GII genotypes, including 1 new GII genotype, were detected. Our results showed that both symptomatic and asymptomatic food handlers were infected with noroviruses.

MATERIALS AND METHODS

Specimens. Fifty-five outbreaks and 35 sporadic cases of norovirus-associated gastroenteritis occurred at food-catering settings during the two winter periods between 10 November 2005 and 9 December 2006 (Fig. 1). Outbreaks were defined as having both (i) two or more food handlers with symptoms of gastroenteritis, i.e., nausea, vomiting, stomachache, diarrhea, or fever, and (ii) two or more specimens that were positive for norovirus by real-time RT-PCR. Sporadic cases were defined as having (i) only one symptomatic food handler and/or (ii) only one specimen positive for norovirus by real-time RT-PCR (even if two or more food handlers were symptomatic). Outbreak settings included nursing care

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771. Fax: 81-42-561-4729. E-mail: ghansman@nih.go.jp.

▽ Published ahead of print on 10 October 2007.

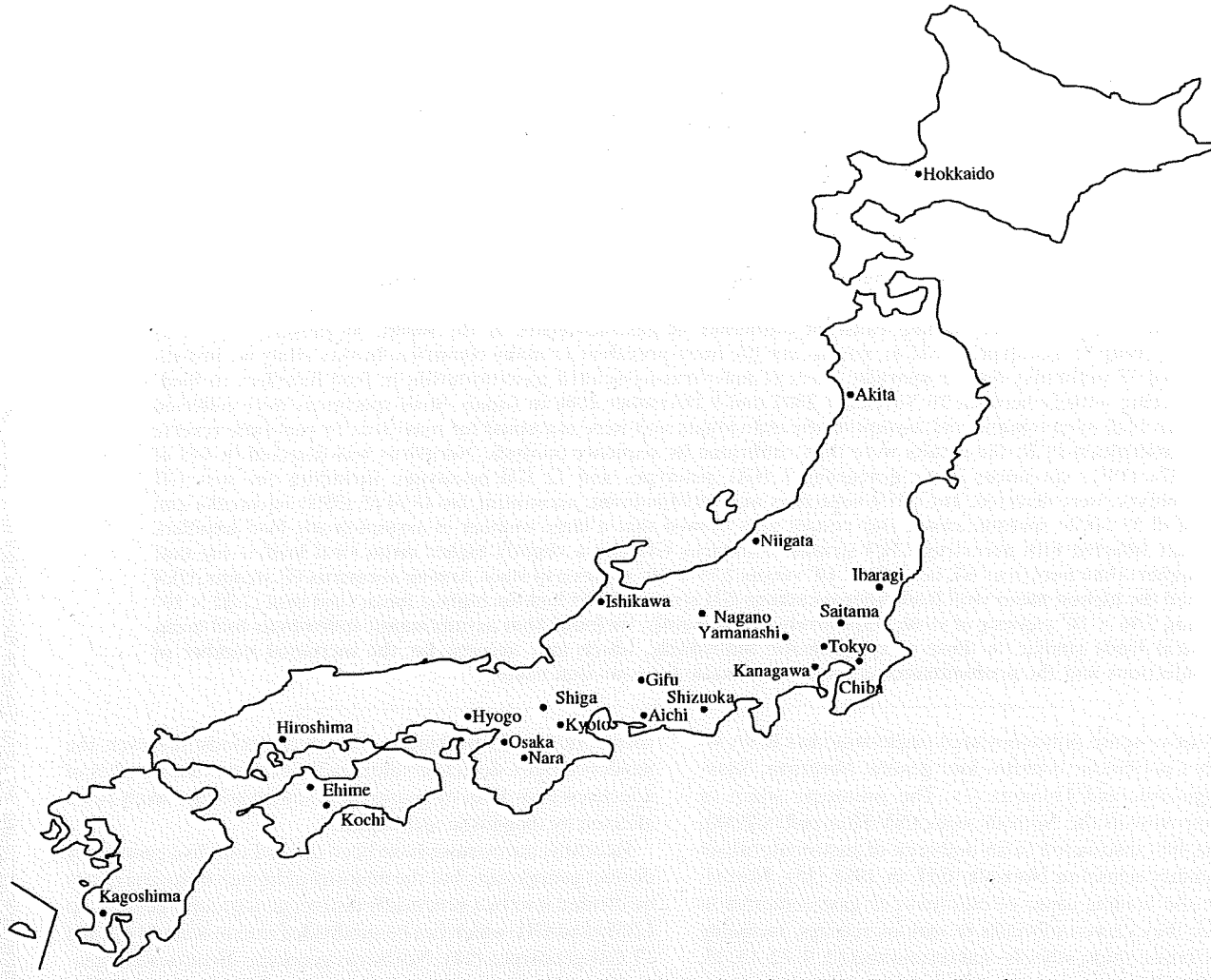

FIG. 1. Map of Japan showing the prefectures/places where the specimens were collected.

centers, fast food establishments, hospitals, school canteens, hotels, restaurants, university cafeterias, and a kindergarten (see Tables 1 and 2). Stool specimens were collected from both symptomatic and asymptomatic employees. In total, 2,376 of approximately 7,000 specimens were examined for norovirus by real-time RT-PCR.

Virus detection. A 10% (wt/vol) stool suspension was prepared with sterilized MilliQ water and centrifuged at 10,000 × *g* for 10 min. The QIAamp viral RNA minivacuum protocol (Qiagen, Hilden, Germany) was used to extract RNA from 140 μl of the clarified supernatant according to the manufacturer's instructions. Briefly, cDNA synthesis was carried out with 10 μl of the RNA in 20 μl of the reaction mixture containing 50 pmol random hexamer (Takara, Tokyo, Japan), 1× Superscript III reverse transcriptase buffer (Invitrogen, Carlsbad, CA), 10 mM dithiothreitol (Invitrogen), 0.4 mM each deoxynucleoside triphosphate (Roche, Mannheim, Germany), 1 U RNase inhibitor (Toyobo, Tokyo, Japan), and 10 U Superscript reverse transcriptase III (Invitrogen). RT was performed at 37°C for 15 min, followed by 50°C for 1 h. Real-time RT-PCR was performed as previously described, and the cutoff for positive norovirus specimens was set at >10 copies per well (13).

Sequencing and phylogenetic analysis. Conventional RT-PCR was carried out to sequence the real-time RT-PCR-positive specimens. Briefly, for norovirus GI PCR, primers G1SKF and G1SKR were used, and for norovirus GII PCR, primers G2SKF and G2SKR were used (17). RT-PCR products were excised from the gel and purified with the QIAquick gel extraction kit (Qiagen, Germany). Nucleotide sequences were prepared with the BigDye Terminator cycle sequencing kit (version 3.1) and determined with the ABI 3130 sequencer (ABI, Boston, MA). Nucleotide sequences were aligned with Clustal X, and the distances were calculated by Kimura's two-parameter method. Phylogenetic trees with bootstrap analysis from 1,000 replicas were generated by the neighbor-joining method as described previously (14).

Nucleotide sequence accession numbers. The accession numbers for sequences determined in this study are GenBank EF630426 to EF630534.

RESULTS

Specimens and screening for norovirus by real-time RT-PCR. Fifty-five outbreaks and 35 sporadic cases of norovirus-associated gastroenteritis in Japan were examined between 10 November 2005 and 9 December 2006 (Tables 1 and 2; Fig. 1). Most outbreaks occurred at nursing care centers (25 of 55), followed by hospitals (12 of 55), cafeterias (5 of 55), fast food establishments (7 of 55), schools (3 of 55), hotels (2 of 55), and restaurants (1 of 55). Sporadic cases were also found at a number of these settings (Table 2). In total, 2,376 stool specimens were collected from both symptomatic and asymptomatic food handlers at different food-catering settings. These specimens were screened by real-time RT-PCR, and norovirus

TABLE 1. Details of norovirus-associated gastroenteritis outbreaks in Japan

Genogroup and setting no.	Type of setting	Prefecture/ place	Date(s)	Specimen no.[a]	Genotype(s)	Total persons at setting	No. positive/no. symptomatic	No. positive/no. asymptomatic	Total positive
GII									
1	Fast food	Hyogo	27 Feb. 2006	*283**	3	33	2/3	0/30	2
2	Fast food	Osaka	22 Mar. 2006	*304**	3	25	3/3	1/22	4
3	Fast food	Tokyo	2 Dec. 2006	*419**, *420*, 423, 425*	3, 4, 4, 4	467	37/42	24/425	61
4	Fast food	Osaka	9 Dec. 2006	*427**	4	385	6/7	0/14	6
6	Fast food	Shizuoka	23 Feb. 2006	*277**, 286*	14, 14	7	2/2	0/5	2
8	Nursing care center	Kyoto	25 Nov. 2006	*401**, *402**	4, 4	48	2/2	0/0	2
9	Nursing care center	Kyoto	23 to 30 Jan. 2006	*150**, *213**, 151, 152, 191*, 199*, 200	4, 5, 4, 4, 4, 4, 4	121	7/8	3/21	10
10	Hospital	Kochi	11 Jan. 2006	*92**, *315*	5, 4	95	1/2	1/2	2
11	Hospital	Yamaguchi	6 to 18 Mar. 2006	*288**, 296*	5, 5	9	2/2	0/0	2
14	Nursing care center	Saitama	21 to 26 Dec. 2005	*70**, 46, 48, 71*	4, 4, 4, 4	78	4/6	3/5	7
15	Nursing care center	Kanagawa	19 Jan. 2006	*143**, 145	4, 4	85	2/3	1/3	3
17	Nursing care center	Tokyo	7 Feb. 2006	*229**, *281*	3, 4	123	4/8	2/9	6
19	Nursing care center	Tokyo	9 Mar. 2006	*290**, 307*	5, 5	92	2/2	0/6	2
21	School	Chiba	6 Dec. 2005	*12*, 16	4, 4	145	0/2	2/12	2
22	Hospital	Nara	14 Jan. 2006	*103*, 104	4, 4	76	0/1	2/7	2
23	Hospital	Tokyo	5 to 11 Jan. 2006	*80*, *136**, 91*	4, 3, 4	235	2/2	1/10	3
29	Nursing care center	Saitama	19 Jan. 2006	*148*, 159	4, 4	78	0/0	2/5	2
30	Cafeteria	Saitama	12 to 20 Dec. 2005	*17**, 18*, 19*, 30*, 31*, 44*, 45*	4, 4, 4, 4, 4, 4, 4	18	11/12	0/0	11
32	Hospital	Shizuoka	25 to 28 Jan. 2006	*177**, 19*	6, 6	56	2/8	0/0	2
34	Cafeteria	Hyogo	24 Dec. 2005	*52**, *53**, *237*	6, 2, 4	59	4/6	4/53	8
38	Cafeteria	Osaka	13 Dec. 2006	*428**, *430**, 429, 431	4, 4, 4, 4	861	29/48	34/813	63
43	Cafeteria	Tokyo	25 Jan. to 1 Feb. 2006	*174**, 205*	3, 3	21	2/2	2/19	4
44	Fast food	Tokyo	20 Mar. 2006	*300**	9	16	1/1	1/15	2
46	Fast food	Tokyo	25 Jan. 2006	*175*	3	36	2/4	4/32	6
47	Restaurant	Tokyo	17 Feb. to 22 Mar. 2006	*268**, 269, 306*	5, 5, 5	41	6/6	2/35	8
48	Hotel	Tokyo	10 to 16 Jan. 2006	*84**, *106**, *233**, 105*, 228*, 270*	3, 6, 5, 3, 3, 5	42	6/11	7/31	13
49	Nursing care center	Aichi	10 to 16 Feb. 2006	*234*, *239*, *256**	4, 4, 3	110	1/1	2/14	3
51	Hospital	Yamanashi	16 to 23 Jan. 2006	*111*, *112**, *114**, 113, 154*	4, 3, 3, 4, 3	156	3/3	2/8	5
54	Hospital	Osaka	5 Dec. 2006	*413**, 414*	4, 4	52	2/2	0/0	2
55	Hospital	Hiroshima	2 Dec. 2005	*11**	3	185	2/4	1/18	3
56	Hospital	Kyoto	27 Nov. 2006	*403**, 405*, 406*	4, 4, 4	78	3/3	0/0	3
57	Hospital	Hyogo	10 Nov. 2005	*1**, 4	4, 4	68	1/2	1/4	2
60	Hospital	Kanagawa	13 Feb. 2006	*241**, 243	4, 4	162	3/4	2/11	5
62	Nursing care center	Tokyo	16 to 24 Jan. 2006	*115**, *118**, *161**, *209**, 162*	4, 4, 10, 6, 10	93	33/41	0/0	33
63	Nursing care center	Tokyo	16 to 19 Dec. 2005	*28*, 43	4, 4	79	0/0	2/8	2
64	Nursing care center	Hyogo	10 Jan. 2006	*86**, 87*, 120*	8, 8, 8	135	3/3	0/14	3
65	Nursing care center	Kanagawa	17 Dec. 2005	*33**, 34*, 35*, 36	4, 4, 4, 4	92	3/5	1/11	4
66	Nursing care center	Ishikawa	16 to 24 Jan. 2006	*127**, 128*, 131*, 132*, 138*, 166*, 167*, 170*, 172*	3, 3, 3, 3, 3, 3, 3, 3, 3	65	9/18	0/0	9
68	Nursing care center	Aichi	16 Dec. 2005	*10**, *15*, 23*, 24, 64*, 65*	6, 4, 6, 4, 6, 6	109	30/35	2/3	32
70	Nursing care center	Chiba	13 Jan. 2006	*101**, 135*	4, 4	36	2/3	0/0	2
71	Nursing care center	Gifu	5 Jan. 2006	*77**	4	85	1/1	1/9	2
73	Nursing care center	Ibaragi	23 Jan. 2006	*156**	3	68	8/11	0/0	8
74	Nursing care center	Shizuoka	16 Dec. 2005	*27**, 50*	4, 4	74	2/4	0/2	2
75	Nursing care center	Hokkaido	18 Mar. 2006	*299*	New	36	1/3	1/6	2
78	Nursing care center	Akita	27 Mar. 2006	*313*, 314	4, 4	96	1/3	2/6	3
79	Nursing care center	Ishikawa	6 to 23 Feb. 2006	*224**	1	55	1/2	1/7	2
80	Nursing care center	Kagoshima	14 Feb. 2006	*251**, 250*, 253*, 278*	2, 2, 2, 2	101	4/5	2/3	6
81	Hospital	Hokkaido	19 to 24 Dec. 2005	*39**, 41*, 42*, 58*, 59*, 60*, 62*	4, 4, 4, 4, 4, 4, 4	65	7/10	2/4	9
83	Nursing care center	Shizuoka	10 to 18 Jan. 2006	*90**, 89, 98*, 126*, 142*	1, 1, 1, 1, 1	85	4/4	2/3	6
84	Hotel	Tokyo	2 Dec. 2006	*410*, 409	4, 4	85	4/4	2/81	6
GI									
101	School	Shizuoka	28 Dec. 2005 to 5 Jan. 2006	*1**, 2, 3, 5, 6*	4, 4, 4, 4, 4	132	2/2	3/6	5
103	School	Kyoto	13 Jan. 2006	*8**, 9*, 10*	8, 8, 8	33	3/5	0/0	3
104	Nursing care center	Tokyo	18 to 23 Jan. 2006	*12**, 14	14, 14	76	1/2	2/6	3
105	Nursing care center	Tokyo	1 to 7 Feb. 2006	*17*, 19, 20*, 21*, 23*, 24*	3, 3, 3, 3, 3, 3	98	3/5	3/12	6
106	Cafeteria	Tokyo	18 to 27 Feb. 2006	*28**, 30*	14, 14	41	5/6	3/35	8
Total						5,802	281/384	133	414

[a] Specimen numbers in italics are shown on the phylogenetic trees. Asterisks indicate symptoms of gastroenteritis.

TABLE 2. Details of sporadic cases of norovirus-associated gastroenteritis in Japan

Genogroup	Setting no.	Type of setting	Prefecture/ place	Date(s)	Specimen no.[a]	Genotype	Total persons at setting	No. positive/no. symptomatic	No. positive/no. asymptomatic	Total positive
GII	5	Fast food	Shizuoka	23 Feb. 2006	*276**	10	7	1/1	0/6	1
	7	Hospital	Kochi	28 Jan. 2006	*195**	4	6	1/1	0/0	1
	12	Meat shop	Saitama	12 Jan. 2006	*97**	7	1	1/1	0/0	1
	13	Fish shop	Tokyo	1 Dec. 2006	*407**	3	1	1/1	0/0	1
	16	Nursing care center	Tokyo	23 Jan. 2006	*153**	4	55	1/1	0/3	1
	18	Nursing care center	Kanagawa	1 Feb. 2006	*204**	4	52	1/1	0/0	1
	20	Hospital	Saitama	25 Jan. 2006	*173**	7	69	1/1	0/2	1
	24	University	Osaka	12 Jan. 2006	*94**	2	8	1/2	0/6	1
	25	Cafeteria	Tokyo	5 Jan. 2006	*79**	4	21	1/5	0/0	1
	26	Cafeteria	Osaka	12 Jan. 2006	*93**	4	13	1/1	0/12	1
	27	Nursing care center	Shiga	26 Dec. 2005	*67**	6	82	1/1	0/5	1
	28	Nursing care center	Saitama	17 Mar. 2006	*293**	3	45	1/1	0/0	1
	31	Cafeteria	Tokyo	19 Jan. 2006	*147**	4	35	1/1	0/0	1
	33	Cafeteria	Osaka	10 Feb. 2006	*235**	3	13	1/1	0/12	1
	35	School	Tokyo	16 Jan. 2006	*110**	4	89	1/1	0/0	1
	36	Fish shop	Shizuoka	20 Feb. 2006	*236**	3	1	1/1	0/0	1
	37	Cafeteria	Shizuoka	29 Dec. 2005	*74**	3	225	1/5	0/0	1
	39	Nursing care center	Tokyo	14 Mar. 2006	*292**	3	8	1/1	0/0	1
	40	Nursing care center	Shizuoka	16 Dec. 2005	*32**	4	6	1/1	0/0	1
	41	School	Shizuoka	14 Dec. 2005	*21**	3	5	1/1	0/0	1
	42	Kindergarten	Shizuoka	24 Dec. 2005	*57**	4	6	1/3	0/0	1
	45	Restaurant	Tokyo	17 Feb. 2006	*260**	5	16	1/2	0/14	1
	50	Nursing care center	Nigata	30 Jan. 2006	*197*	4	8	1/1	0/0	1
	52	Cafeteria	Shizuoka	6 Jan. 2006	*83**	4	6	1/1	0/0	1
	53	Hospital	Hokkaido	2 Feb. 2006	*210**	10	81	1/1	0/5	1
	58	University	Osaka	5 Jan. 2006	*81**	2	8	1/1	0/0	1
	59	Hospital	Akita	30 Jan. 2006	*196**	4	65	1/1	0/4	1
	61	Hospital	Hokkaido	1 Dec. 2005 to 24 Jan. 2006	*9**	4	18	1/1	0/6	1
	67	Nursing care center	Kanagawa	26 Dec. 2005 to 10 Jan. 2006	*85**	3	8	1/1	0/7	1
	69	Nursing care center	Ibaragi	24 Nov. 2006	*404**	4	6	1/1	0/0	1
	72	Nursing care center	Osaka	24 Dec. 2005	*56**	6	52	1/2	0/11	1
	76	Nursing care center	Nagano	30 Dec. 2005	*75**	3	52	1/2	0/4	1
	77	Nursing care center	Aichi	17 Dec. 2005	*37**	4	85	1/2	0/0	1
	82	Hospital	Ehime	2 Dec. 2006	*411**	4	5	1/1	0/0	1
GI	102	School	Shizuoka	11 Jan. 2006	*7**	4	8	1/1	0/0	1
Total							1,166	30		

[a] Italicized numbers indicate that specimens are shown on the phylogenetic trees. Asterisks indicate symptoms of gastroenteritis.

was detected in 449 of 2,376 (19%) specimens. Real-time RT-PCR can distinguish between norovirus GI and GII sequences, and both GI and GII sequences were detected. Twenty-six of 2,376 (1%) stool specimens were positive for norovirus GI sequences, and 423 of 2,376 (18%) were positive for norovirus GII sequences. Noroviruses were detected in specimens from both symptomatic and asymptomatic food handlers (see Tables 1 and 2).

Genotyping and phylogenetic analysis of norovirus. To confirm the positive real-time RT-PCR results and determine the genotypes, we reamplified and sequenced the partial capsid gene. We simplified the phylogenetic trees to include only unique sequences (italicized in Table 1, specimen number column); that is, when two or more sequences from the same outbreak had 100% nucleotide similarity, we named a single consensus sequence, and if a sequence had one or more nucleotide mismatches with others in the same outbreak, we gave the sequence a distinct name. All 26 norovirus GI-positive specimens (from 25 outbreak cases and 1 sporadic case) were sequenced, and these clustered into four distinct genotypes: GI/3, GI/4, GI/8, and GI/14 (Fig. 2). One or more norovirus GII-positive specimens in each setting were confirmed by RT-PCR and sequenced. The GII sequences belonged to 12 genotypes, including one new genotype: GII/1, GII/2, GII/3, GII/4, GII/5, GII/6, GII/7, GII/8, GII/9, GII/10, GII/14, and GII/New (Fig. 3).

Molecular epidemiology of outbreaks. Norovirus GI sequences were detected in 5 of 55 (9%) outbreaks (Table 1). We found 14 of 20 (70%) symptomatic food handlers and 11 of 59 (19%) asymptomatic food handlers positive for norovirus GI strains. Sequence analysis showed that each of the five norovirus GI-associated outbreaks was caused by a single norovirus GI strain; that is, two or more sequences from the same outbreak shared 100% nucleotide similarity. Norovirus GII sequences were detected in 50 of 55 (91%) outbreaks. We found 267 of 364 (73%) symptomatic food handlers and 122 of 1,786 (7%) asymptomatic food handlers positive for norovirus GII strains. In 37 of 50 norovirus GII-associated gastroenteritis

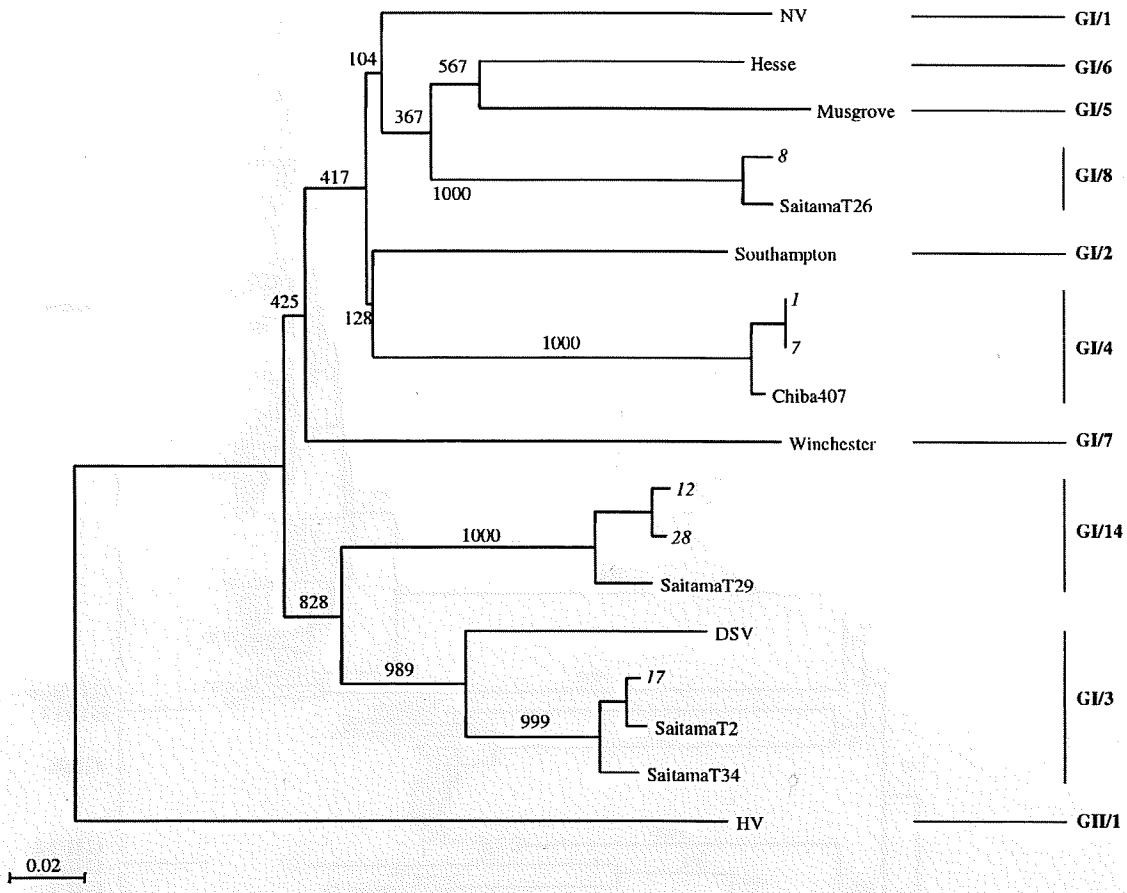

FIG. 2. Phylogenetic tree of the norovirus GI sequences detected in this study (italicized specimen numbers). Norovirus nucleotide sequences were constructed with the partial N-terminal capsid region (14) by using the norovirus Hawaii GII sequence as an outgroup. We simplified the tree to include only unique sequences; that is, when two or more sequences in the same outbreak had 100% nucleotide similarity, we named a single consensus sequence, and if a sequence had one or more nucleotide mismatches with others in the same outbreak, we gave the sequence a distinct name. The numbers on the branches are the bootstrap values for the clusters. Bootstrap values of 950 or higher were considered statistically significant for the grouping (14).

outbreaks, a single norovirus GII strain was assumed to be responsible, since two or more sequences from the same outbreak shared 100% nucleotide similarity. In the remainder of the GII-associated outbreaks (13 of 50 outbreaks; settings 3, 8, 9, 10, 17, 23, 34, 38, 48, 49, 51, 62, and 68), several norovirus GII sequences were detected; that is, there were one or more nucleotide changes or different genotypes (Table 1). For example, in the norovirus GII-associated setting 48 (Table 2), we detected three norovirus genotypes (GII/3, GII/5, and GII/6) in stool specimens collected from different symptomatic food handlers in the same hotel between 10 and 16 January 2006, whereas for setting 8, a nursing care center, we detected different GII/4 sequences on 25 November 2006 (specimens 401 and 402) (Fig. 3). Interestingly, symptomatic and asymptomatic food handlers were positive by real-time RT-PCR in 9 of these 13 mixed norovirus GII outbreaks (settings 3, 9, 10, 17, 23, 34, 49, 51, and 68). For example, in the norovirus GII-associated setting 34 (Table 1), we detected three norovirus genotypes (GII/6, GII/4, and GII/2); the former two sequences (specimens 52 and 53) were detected in specimens from symptomatic food handlers, and the latter sequence (specimen 237) was detected in a specimen from an asymptomatic food handler.

GII/4 sequences were predominant, accounting for 20 of 55 (36%) outbreaks, excluding the outbreaks with multiple norovirus genotypes (Fig. 4). A considerable number of outbreaks (7 of 55) were also caused by strains belonging to GII/3, followed by GII/5, which caused 3 outbreaks. Interestingly, in 12 of 13 mixed-GII-genotype outbreaks (settings 3, 8, 9, 10, 17, 23, 34, 38, 49, 51, 62, and 68), we always detected a norovirus GII/4 sequence (Table 1).

Molecular epidemiology of sporadic cases. Among 35 sporadic cases, norovirus GI sequences were detected in 1 case (3%), which was caused by a GI/4 strain (Table 2). Norovirus GII sequences were detected in 34 of 35 (97%) sporadic cases. The majority of the GII sporadic cases were caused by GII/4 strains (16 of 35), followed by GII/3 (9 of 35). Interestingly, for sporadic cases, only food handlers with symptoms of gastroenteritis were positive for norovirus; 35 of 49 (71%) were positive for GII strains, and 1 of 1 (100%) was positive for GI strains.

Genetic analysis. The partial capsid sequence was used to describe the genetic diversity of the norovirus sequences. All

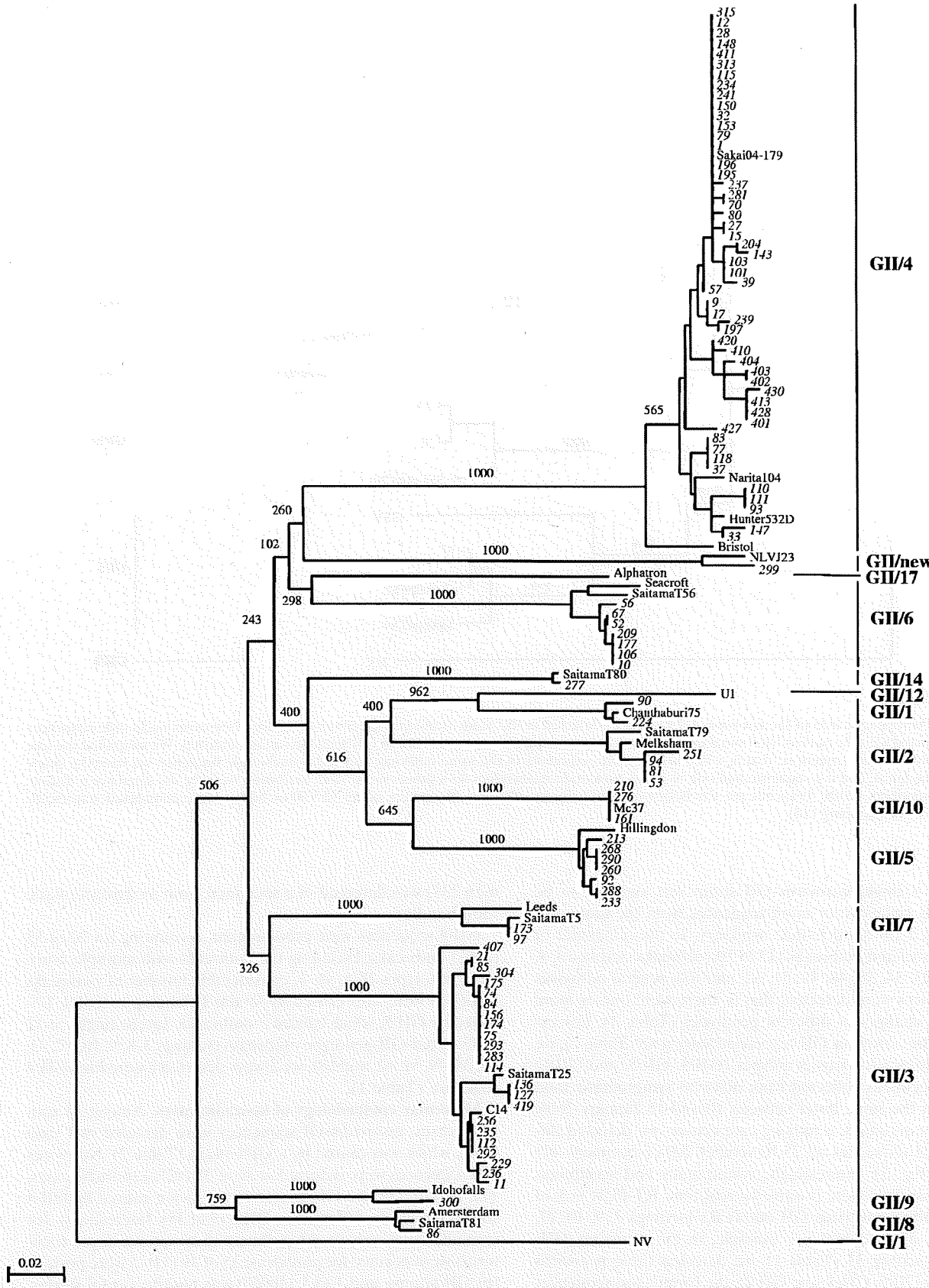

FIG. 3. Phylogenetic tree of the norovirus GII sequences detected in this study (italicized specimen numbers). Norovirus nucleotide sequences were constructed with the partial N-terminal capsid region (14) by using the Norwalk virus GI sequence as an outgroup.

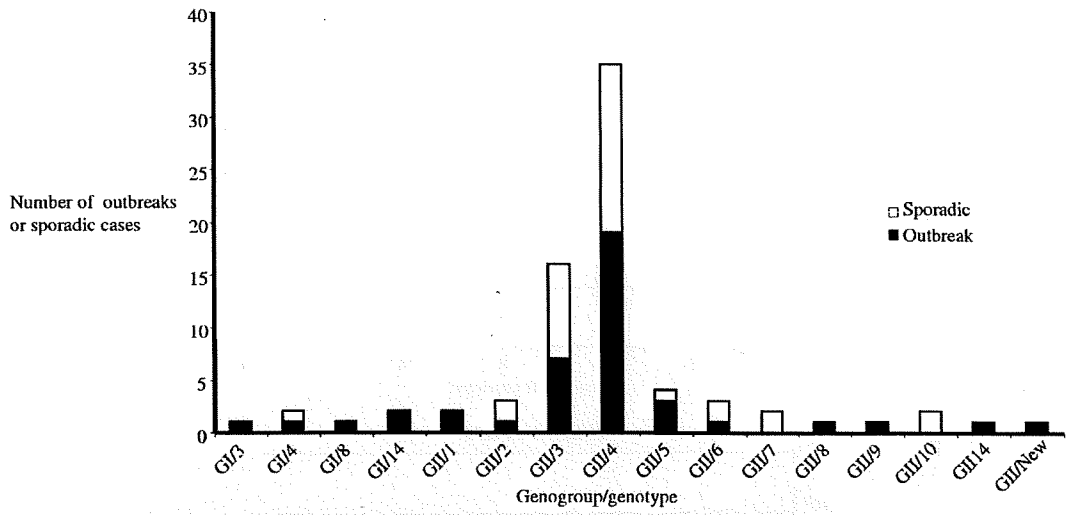

FIG. 4. Number of outbreaks caused by each genotype. Only outbreaks due to a single genotype are included. For example, norovirus GII-associated outbreak 8 had two GII/4 sequences and was therefore considered to be a GII/4-associated outbreak, but the GII-associated outbreak 9 had GII/4 and GII/5 sequences, so the cause of the outbreak was unknown, and it was excluded from the figure.

sequences detected in this study closely matched other, published sequences (Fig. 2 and 3). The GII/4 sequences shared >95% nucleotide similarity, although there was noticeable subclustering within the GII/4 genotype (Fig. 3). Norovirus GII/3 also appeared to have subclusters, while the GII/3 sequences shared >96% nucleotide similarity (Fig. 3). Of interest was the detection of a novel GII genotype (GII/New; sequence 299). As shown in Fig. 5, the amino acid start sequence for the capsid was MRM, whereas all other norovirus GII sequences had MKM. To further investigate this finding, we sequenced the entire capsid gene. Only one other full-length capsid sequence (norovirus strain NLVJ23; accession number GenBank AY130762) closely matched the norovirus 299 sequence, with 99.3% amino acid identity over the entire capsid gene. Interestingly, norovirus NLVJ23 also had the unusual amino acid start sequence of MRM for the capsid. The N-terminal regions of the capsids of these two sequences were quite unlike that of any other norovirus GII sequence. In addition, both norovirus 299 and NLVJ23 had an amino acid insertion at the 10th residue, whereas no other norovirus GII sequences had any insertions or deletions within the first 149 amino acids of the capsid gene (Fig. 5).

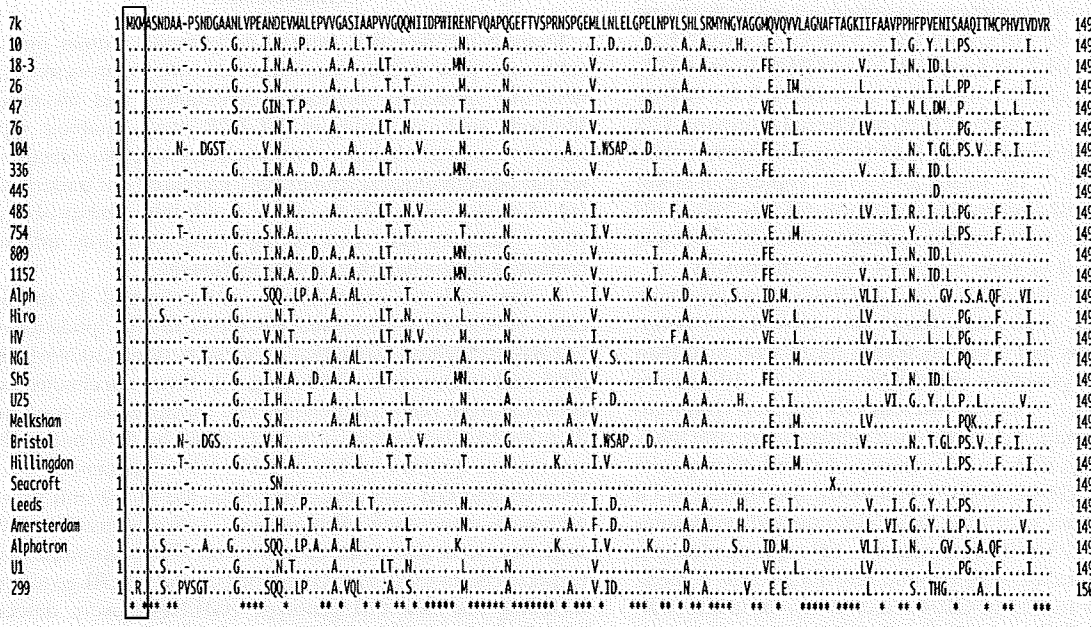

FIG. 5. Amino acid alignment of the partial N-terminal-region capsid sequences of the norovirus GII strains. The predicted highly conserved MKM capsid start sequence is boxed. Asterisks indicate conserved amino acids among these capsid sequences.

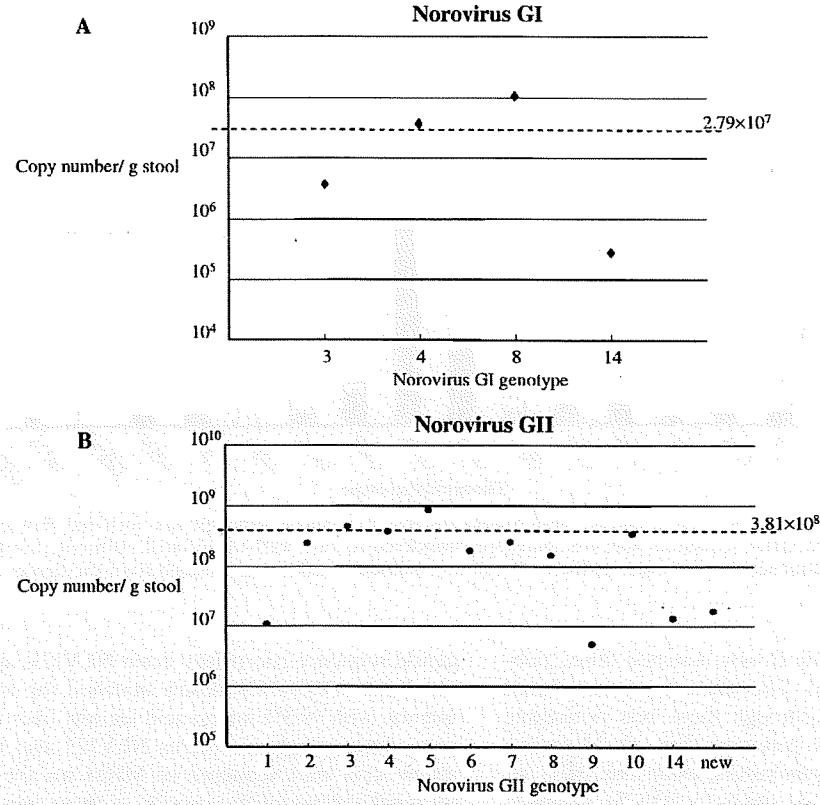

FIG. 6. Viral loads for GI and GII noroviruses. Dotted lines represent the mean for each genogroup.

Analysis of norovirus loads. The real-time RT-PCR results were used to analyze the viral loads by genotype and genogroup (Fig. 6). All positive sequences (not just the consensus sequences shown in the trees) were used for this analysis. The numbers of positive specimens examined for each genotype were as follows: 6 for GI/3, 6 for GI/4, 3 for GI/8, 4 for GI/14, 6 for GII/1, 7 for GII/2, 35 for GII/3, 96 for GII/4, 13 for GII/5, 11 for GII/6, 2 for GII/7, 3 for GII/8, 1 for GII/9, 3 for GII/10, 2 for GII/14, and 1 for GII/new (Fig. 6). Overall, norovirus GII had a slightly higher mean viral load (1 log unit higher) than norovirus GI (3.81×10^8 versus 2.79×10^7 copies/g of stool) (Fig. 6). The highest viral load for norovirus GI was 2.02×10^8 copies/g of stool for GI/4, and the highest viral load for GII was 7.96×10^9 copies/g of stool for GII/4 (data not shown).

A comparison between the mean viral loads of symptomatic and asymptomatic individuals found that GI-infected symptomatic individuals had a slightly higher mean viral load than GI-infected asymptomatic individuals (4.43×10^7 versus 3.79×10^6 copies/g of stool), whereas GII-infected symptomatic and asymptomatic individuals had similar mean viral loads (3.31×10^8 versus 5.53×10^8 copies/g of stool). Our results also showed that GI/4-infected symptomatic individuals had a slightly higher mean viral load than GI/4-infected asymptomatic individuals (6.73×10^7 versus 6.34×10^6 copies/g of stool), whereas GII/4-infected symptomatic individuals had a mean viral load similar to that of GII/4-infected asymptomatic individuals (2.17×10^8 versus 6.58×10^8 copies/g of stool). Comparisons between the other genotypes were difficult to perform because the asymptomatic individuals were not infected with those genotypes (data not shown).

DISCUSSION

Despite the fact that human noroviruses remain uncultivable, the past decade has witnessed vast improvements in norovirus detection methods, surveillance, and awareness. Methods such as real-time RT-PCR have enabled rapid, broadly reactive, and highly sensitive screening. Numerous molecular epidemiological studies have increased our understanding of these viruses. On the other hand, the number of norovirus infections still remains high, and norovirus infection remains a major health problem worldwide.

In this study we examined 55 outbreaks and 35 sporadic cases of norovirus-associated gastroenteritis in food-catering settings throughout Japan that occurred between 10 November 2005 and 9 December 2006 (Fig. 1). Stool specimens were collected from both symptomatic and asymptomatic food handlers so that the transmission route could be determined. Norovirus was detected in 449 of 2,376 (19%) specimens. Norovirus GI and GII sequences were detected in 9% and 91% of outbreaks, respectively (Tables 1 and 2). Norovirus GI and GII sequences were also detected in 3% and 97% of sporadic cases, respectively. In total, four GI genotypes and 12 GII genotypes, including one new GII genotype (GII/New), were detected during the study period (Fig. 2 and 3).

All of the norovirus GI-associated outbreaks were caused by

a single norovirus GI genotype, whereas a single norovirus GII genotype was detected in 38 of 50 norovirus GII-associated outbreaks. In the remainder of the norovirus GII-associated outbreaks (13 of 50 outbreaks), multiple norovirus GII sequences with mismatches or different genotypes were detected (Tables 1 and 2). The GII/4 strains appeared to be the dominant cause of the outbreaks. GII/4 strains were detected in 20 of 55 (36%) outbreaks, followed by GII/3, which was detected in 7 of 55 outbreaks. We also found that noroviruses belonging to GII/4 were the dominant cause of outbreaks in Taiwan (32). In a number of norovirus GII-associated outbreaks (9 of 50 outbreaks), different norovirus genotypes were detected in specimens from symptomatic and asymptomatic food handlers from the same food-catering setting. Interestingly, many of the asymptomatic food handlers were also positive for a norovirus GII/4 sequence, although we cannot be certain whether the subject(s) later developed symptoms (Table 1). Nevertheless, taken as a whole, these results have shown that asymptomatic infections were widespread in the food-catering industry in Japan at the time of the study. Recently, excretion of norovirus by symptomatic and asymptomatic individuals during a hospital outbreak of gastroenteritis where a GII/4 strain was dominant has been described (6). At present, norovirus GII/4 strains are the most prevalent in many countries (4, 8, 27, 28). What is more, variant GII/4 sequences, i.e., those differing by approximately 5% of amino acids, were speculated to be more virulent and part of the reason for the increased number of infections worldwide (8). Clearly, norovirus GII/4 strains are widespread, although they may not always cause symptoms, which may account for the increased number of infections via a "silent" (that is, asymptomatic) transmission route. GII/4 strains were also the dominant cause of the sporadic cases; they were detected in 16 of 35 (46%) sporadic cases. This result suggests that the GII/4 strains are an important cause of both outbreaks and sporadic occurrences of gastroenteritis.

Noroviruses can be transmitted by the fecal-oral route through person-to-person contact and by food- and waterborne infections (2, 3, 12). In Japan, oyster-associated gastroenteritis is a major problem, and it is not unusual to detect multiple norovirus genotypes in an oyster-associated outbreak (14). In this study, multiple norovirus GII sequences were detected in the same outbreak, but norovirus GI and GII sequences were not detected in the same outbreak. The low infectious dose (22) and prolonged shedding (11) of norovirus makes transmission almost certain, although we could not be certain whether foods were contaminated. Mean viral loads of GI and GII were found to be 2.79×10^7 and 3.81×10^8 copies/g of stool, respectively (Fig. 6). Similar viral loads were found for infected symptomatic and asymptomatic individuals, indicating the potential hazard of these highly contagious viruses. In a recent study in which the number of norovirus cDNA copies per gram of stool specimen was analyzed, a discrepancy was found between the different norovirus genogroups. Chan et al. found median viral loads of 8.4×10^5 and 3.0×10^8 copies/g of stool specimen for norovirus GI and GII, respectively, and speculated that the higher viral loads of GII strains were due to their higher transmissibility (5). Of note, our results showed that GII/4 strains had the highest mean viral load overall (7.96×10^9 copies/g of stool), further increasing the clinical importance of this dominating genotype.

Norovirus GII capsid sequences are highly conserved at the N-terminal region and, to the best of our knowledge, share an identical amino acid start sequence, MKM. In this study, we identified an atypical norovirus GII capsid amino acid start sequence, MRM, in the norovirus 299 sequence (Fig. 5). What is more, the MRM amino acid residues did not match other norovirus genogroups, and only one other closely matching sequence was found in the database (norovirus strain NLVJ23). Interestingly, the N-terminal capsid region is highly conserved in all norovirus GI sequences and shares the same amino acid start sequence, MMM. This suggests that either norovirus 299 and NLVJ23 may not belong to norovirus GII or the N-terminal capsid region is not as highly conserved in each genogroup as previously anticipated.

In conclusion, we found that norovirus infections were a common cause of gastroenteritis in the food-catering industry in Japan. Our results have also shown that asymptomatic infections with noroviruses, whether with a sequence identical to that infecting a symptomatic food handler or with a distinct sequence, were widespread in the food-catering industry. Much work is needed to curb the burden of this disease and reduce its transmission. A simple workplace policy that will protect ill workers and allow for paid leave may not be sufficient to stop transmission, since asymptomatic food handlers may continue to work.

ACKNOWLEDGMENTS

This work was supported in part by a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan and by a grant for Research on Health Science Focusing on Drug Innovation from The Japan Health Science Foundation.

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Antigenic Diversity of Human Sapoviruses

Grant S. Hansman,* Tomoichiro Oka,* Naomi Sakon,† and Naokazu Takeda*

Sapovirus (SaV) is a causative agent of gastroenteritis. On the basis of capsid protein (VP1) nucleotide sequences, SaV can be divided into 5 genogroups (GI–GV), of which the GI, GII, GIV, and GV strains infect humans. SaV is uncultivable, but expression of recombinant VP1 in insect cells results in formation of viruslike particles (VLPs) that are antigenically similar to native SaV. In this study, we newly expressed SaV GII and GIV VLPs to compare genetic and antigenic relationships among all human SaV genogroups. Hyperimmune antiserum samples against VLPs reacted strongly with homologous VLPs. However, several antiserum samples weakly cross-reacted against heterologous VLPs in an antibody ELISA. Conversely, an antigen ELISA showed that VLPs of SaV in all human genogroups were antigenically distinct. These findings indicate a likely correspondence between SaV antigenicity and VP1 genogrouping and genotyping.

The family *Caliciviridae* contains 4 genera (*Sapovirus*, *Norovirus*, *Lagovirus*, and *Vesivirus*), which include *Sapporo virus*, *Norwalk virus*, *Rabbit hemorrhagic disease virus*, and *Feline calicivirus*, respectively. Sapoviruses (SaVs) and noroviruses (NoVs) are etiologic agents of human gastroenteritis. The prototype strain of human SaV, Sapporo virus, was originally discovered in an outbreak in an orphanage in Sapporo, Japan, in 1977 (*1*). SaV infects children and adults and has been found to cause outbreaks of gastroenteritis in daycare centers, healthcare facilities, and elementary schools. Detection methods include reverse transcription–PCR (RT-PCR), real-time RT-PCR, enzyme immunoassays, and ELISAs (*2–6*). Recently, we detected SaV in untreated wastewater samples, treated wastewater samples, and river samples (*7*).

*National Institute of Infectious Diseases, Tokyo, Japan; and †Osaka Prefectural Institute of Public Health, Osaka, Japan

The SaV genomes are predicted to contain either 2 or 3 main open reading frames (ORF1–3). SaV ORF1 encodes for nonstructural proteins and the major capsid protein (VP1), and ORF2 (VP2) and ORF3 (VP3) encode proteins of yet unknown functions. On the basis of VP1 nucleotide sequences, SaVs have been divided into 5 genogroups (GI–GV), of which GI, GII, GIV, and GV strains infect humans and GIII strains infect porcine species (*8*). SaV genogroups can be further subdivided into genotypes. Recently, we identified several recombinant SaV strains (*8,9*).

Human SaV and NoV strains are uncultivable, but expression of a recombinant subgenomic-like construct (i.e., VP1 to the end of the genome) or VP1 alone in insect or mammalian cells results in the formation of viruslike particles (VLPs) that are morphologically similar to native SaV (*10–16*). However, production of VLPs of SaV remains difficult, usually only resulting in low yields of VLPs compared with norovirus (*10,12,16,17*). Cryoelectron microscopy and x-ray crystallography analyses of NoV VLPs identified the shell (S) and protruding domains (subdomains P1–1, P1–2, and P2) (*18*). Also, Chen et al. described strictly and moderately conserved amino acid residues in the capsid protein among the 4 genera in the family *Caliciviridae* (*13*).

Previously, we reported that SaV GI/1 (strain Mc114) and GV/1 (strain NK24) were antigenically distinct (*5,10*). More recently, we discovered that SaV GI/5 (strain Yokote1) VLPs were antigenically distinct from SaV GI/1 Mc114 and GV/1 NK24 VLPs (*19*). Other than these few studies, little is known about the genetic and antigenic relationships among the 4 human SaV genogroups. For classification of NoV, distinct genotypes have been defined as having bootstrap values >950 (VP1 sequences); at least 14 GI and 17 GII genotypes have been identified (*20*). For SaV, genogroups have only been vaguely defined, mostly

because 2 of them (GIV/1 and GV/1) were only recently identified, few sequences exist in the database, and antigenic relationships among all genogroups are unknown. In addition, genetic recombination was only recently discovered and appears to be common within the genus *Sapovirus*.

The purpose of this study was to examine cross-reactivities among the 4 human SaV genogroups and compare results with those of genetic analysis. For this purpose, 2 other SaV strains, GII/3 Syd53 and GIV/1 Syd3, were expressed and antisera were produced against their purified VLPs. A total of 5 SaV strains (GI/1 Mc114, GI/5 Yokote1, GII/3 Syd53, GIV/1 Syd3, and GV/1 NK24) that include all 4 human genogroups and 2 GI genotypes were compared. Our results show that SaV genogroups were antigenically distinct and corresponded with results of genetic classification on the basis of full-length VP1 nucleotide sequences. Proper genetic classification of SaV strains is required, and a consensus of genogroups and genotypes that represent genetically and antigenically diverse strains, which include recombinant SaV strains, should be established to avoid conflicting grouping.

Materials and Methods

Specimens

Virus-positive stool specimens were collected from several sources. SaV strain Mc114 (GenBank accession no. AY237422) was isolated from an infant hospitalized with acute gastroenteritis in Chiang Mai, Thailand, in 2001 (*21*). SaV strain NK24 (AY646856) was isolated from an infant with gastroenteritis in Nong Khai, Thailand, in 2003 (*22*). SaV strain Yokote1 was isolated from an outbreak of gastroenteritis at a kindergarten in Yokote City, Japan, in 2006 (*19*). SaV strains Syd53 and Syd3 were isolated from infants hospitalized with acute gastroenteritis in Sydney, New South Wales, Australia, in 2001 (*23*). NoV strain Osaka659 was isolated from an outbreak of gastroenteritis in Japan, in 2006 (unpub. data). RNA extraction and RT-PCR were performed as described (*24*).

Sequence Analysis

Nucleotide sequences were determined by using the Terminator Cycle Sequence Kit version 3.1 and an ABI 3130 sequencer (both from Applied Biosytems, Boston, MA, USA). Nucleotide sequences were aligned with ClustalX (www.embl.de/~chenna/clustal/darwin) and the distances were calculated by the Kimura 2-parameter method (*24*). Phylogenetic trees with bootstrap analysis from 1,000 replicas were generated by the neighbor-joining method as described (*20*). Amino acid VP1 secondary structure predictions were made by using PSIPRED secondary structural prediction software (*25*).

Expression of Viruslike Particles

For the expression of VP1 in insect cells, all SaV constructs were designed to begin from the predicted VP1 start AUG codon and included the VP2 and poly(A) sequences. SaV strains Syd53 and Syd3 were cloned as described (*10*) for strains SaV Mc114, NK24, and Yokote1 according to the protocol of the Baculovirus Expression System using Gateway Technology (Invitrogen, Carlsbad, CA, USA). Briefly, strains Syd53 and Syd3 were amplified with specific sense primers Syd53attb1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGAGGGTGTGTCCCACCCAGA-3′)andSyd3attb1(5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGAGGGCAATGGCCTACCCCAGGCTG-3′) and antisense primer TX30SXN (5′-GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′). PCR fragments were purified after electrophoresis on a 1.0% agarose gel. Fragments were cloned into donor vector pDONR201 (Invitrogen) and transferred into a baculovirus transfer vector pDEST8 (Invitrogen).

The recombinant pDEST8 was purified and used to transform *Escherichia coli* DH10Bac-competent cells (Invitrogen), which produced recombinant bacmids (baculovirus shuttle vectors) containing the VP1 gene. Recombinant bacmids were then transfected into Sf9 cells (Riken Cell Bank, Ibaraki, Japan), and recombinant baculoviruses were isolated. Recombinant baculoviruses were used to infect $\approx 3 \times 10^6$ confluent Tn5 cells (Invitrogen) at a multiplicity of infection of 5–10 in 1.5 mL of Ex-Cell 405 medium (JRH Biosciences, Lenexa, KS, USA), and the infected cells were incubated at 26°C. The culture medium was harvested 5–6d postinfection, centrifuged for 10 min at 3,000× *g*, and further centrifuged for 30 min at 10,000× *g*. VLPs were concentrated by ultracentrifugation for 2 h at 45,000 rpm at 4°C (Beckman TLA-55 rotor; Beckman Coulter, Fullerton, CA, USA), and resuspended in 30 μL of Grace's medium. Samples were examined for VLP formation by electron microscopy as described (*10*), and large-scale production of VLPs was performed as described (*24*).

Antibody Production

Hyperimmune sera to newly expressed VLPs of SaV (Syd53 and Syd3) were prepared in rabbits and guinea pigs. The first subcutaneous injection was performed with purified VLPs (≈10 μg) in Freund complete adjuvant. After 3 weeks, the animals received 1 booster injection (intravenously in rabbits and subcutaneously in guinea pigs) of 10 μg of VLPs without adjuvant. Blood was collected from the animals 1 week after their last booster injection.

Antibody ELISA

Cross-reactivities among antiserum samples against SaV were examined by using an antibody ELISA with hyperimmune rabbit antibodies against VLPs. Briefly, wells of 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were each coated with 100 μL of purified VLPs (≈1.0 μg/ mL in carbonate-bicarbonate buffer, pH 9.6) (Sigma, St. Louis, MO, USA) and incubated overnight at 4°C. Wells were washed twice with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) and blocked with PBS containing 5% skim milk (PBS-SM) for 1 h at room temperature. Wells were then washed 4 times with PBS-T, 100 μL of 2-fold–diluted hyperimmune rabbit antibodies from an initial concentration of 1:500 in PBS-T-SM was added to each well, and the plates were incubated for 1 h at 37°C. Wells were then washed 4 times with PBS-T, and 100 μL of a 1:1,000 dilution of horseradish peroxidase–conjugated goat antirabbit immunoglobulin G diluted in PBS-T-SM was added to each well. Plates were incubated for 1 h at 37°C. Wells were then washed 4 times with PBS-T, and 100 μL of substrate (*o*-phenylenediamine) and H_2O_2 were added to each well, and the plates were left in the dark for 30 min at room temperature. The reaction was stopped by the addition of 50 μL of 2N H_2SO_4 to each well, and the absorbance was measured at 492 nm (A_{492}). The optical density (OD) cutoff point was determined to be 0.15, which was equal to 3 times the mean OD of preimmune serum (*5*).

Antigen ELISA

Cross-reactivities among VLPs were also examined by using an antigen ELISA. Briefly, wells were coated with 100 μL of a 1:8,000 dilution of hyperimmune rabbit antiserum diluted in PBS (except for Syd3, for which a 1:3,000 dilution was used), and the plates were incubated overnight at 4°C. Wells were washed 4 times with PBS-T and blocked with PBS-SM for 1 h at room temperature. Wells were then washed 4 times with PBS-T, 100 μL of VLPs (≈1.0 μg/mL in carbonate-bicarbonate buffer, pH 9.6) (Sigma) was added to duplicate hyperimmune rabbit wells, and the plates were incubated for 1 h at 37°C. Wells were then washed 4 times with PBS-T, 100 μL of a 1:8,000 dilution of hyperimmune guinea pig antibody diluted in PBS-T-SM was added to each well (except for Syd3, which used a 1:3,000 dilution), and the plates were incubated for 1 h at 37°C. Wells were washed 4 times with PBS-T, and 100 μL of a 1:1,000 dilution of horseradish peroxidase–conjugated rabbit antiguinea pig immunoglobulin G diluted in PBS-T-SM was added to each well. The plates were then processed as described above. On the basis of our previous study, a specimen with an A_{492} (P – N) >0.1 and a P/N ratio >1.34 (where P is hyperimmune antiserum and N is preimmune antiserum) was considered significantly positive (*4*).

Results

Sequence Analysis

The sequence of the 3′ end of the genome (≈2,600 nt), i.e., VP1 to poly(A), for the newly expressed SaV strains (Syd53 and Syd3) was determined. Genetic analysis was performed with only complete VP1 sequences, which included sequences from our epidemiologic studies and other sequences available on the database (Figure 1). Five SaV GI and 6 GII genotypes were observed, but only 1 genotype for SaV GIV and 1 for GV was found. This result suggests that SaV GI and GII strains were more genetically diverse, prevalent, or more virulent than SaV GIV and GV strains. However, because the SaV GIV and GV strains were only recently detected (*26*,*27*), this result may reflect only the specificity and sensitivity of the detection methods used. On the basis of our previous classifications, SaV Mc114 and Yokote1 sequences both belonged to GI, but to different genotypes, GI/1 and GI/5, respectively; Syd53 be-

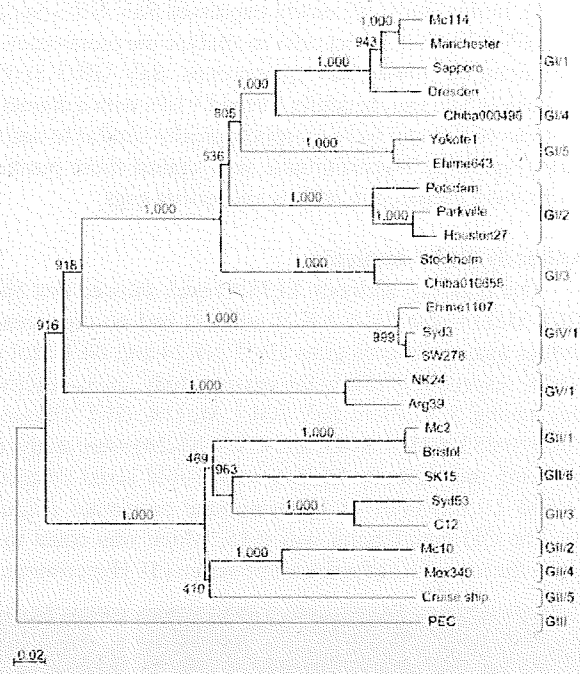

Figure 1. Phylogenetic tree of sapoviruses on the basis of entire nucleotide sequences of the capsid protein. Different genogroups and genotypes are indicated. The numbers on each branch indicate the bootstrap values for the genotype. The scale bar at the lower left represents nucleotide substitutions per site. GenBank accession nos. for reference strains: Arg39, AY289803; Bristol, HCA249939; C12, AY603425; Chiba000496, AJ412800; Chiba010658, AJ606696; Cruise ship, AY289804; Dresden, AY694184; Ehime643, DQ366345; Ehime1107, DQ058829; Houston27, U95644; Manchester, X86560; Mc2, AY237419; Mc10, AY237420; Mc114, AY237422; Mex340, AF435812; NK24, AY646856; Parkville, U73124; PEC, AF182760; Potsdam, AF294739; Sapporo, U65427; SK15, AY646855; Stockholm, AF194182; SW278, DQ125333; Syd3, DQ104357; Syd53, DQ104360; and Yokote1, AB253740.

Table 1. Nucleotide similarity (values below blank diagonal) and amino acid identity (values above blank diagonal) of complete capsid (VP1) sequences of sapovirus strains*

Strain	Mc114 (GI/1)	Yokote1 (GI/5)	Syd53 (GII/3)	Syd3 (GIV/1)	NK24 (GV/1)
Mc114 (GI/1)		79	46	52	51
Yokote1 (GI/5)	76.5		46.8	50.3	50.9
Syd53 (GII/3)	56.1	56.9		48.3	48.6
Syd3 (GIV/1)	58.1	57.4	55.9		54.2
NK24 (GV/1)	58.2	57.3	56.4	58.6	

*Values are percentages.

longed to GII/3; Syd3 belonged to GIV/1; and NK24 belonged to GV/1. SaV GI/1 Mc114 and GI/5 Yokote1 VP1 sequences shared 76.5% and 79% nucleotide similarity and amino acid identity, respectively (Table 1). The nucleotide similarity and amino acid identity among the genogroups was low, i.e., <60% (Table 1).

Expression of VP1

We previously expressed SaV GI/1 Mc114, GI/5 Yokote1, and GV/1 NK24 in insect cells, which resulted in the formation of VLPs morphologically similar to native SaV (*5*,*10*). In this study, we newly expressed SaV GII/3 Syd53 and GIV/1 Syd3 in insect cells to analyze the cross-reactivity among all human SaV genogroups. SaV GII/3 Syd53 and GIV/1 Syd3 successfully formed VLPs with a diameter of 41 to 46 nm and were morphologically similar to native SaV (Figure 2). Hyperimmune sera against these purified VLPs were prepared in rabbits and guinea pigs.

Antibody ELISA Analysis

Our previous antibody ELISA result showed that SaV GI/1 Mc114 and GV/1 NK24 antisera had no cross-reactivities against heterologous GV/1 NK24 and GI/1 Mc114 VLPs, respectively (*5*). In the current study, cross-reactivities among 5 VLPs of SaV (GI/1 Mc114, GI/5 Yokote1, GII/3 Syd53, GIV/1 Syd3, and GV/1 NK24) were analyzed by using the antibody ELISA with 2-fold serial dilutions (1:500–1:1,024,000) of hyperimmune antiserum (Figure 3). The OD cutoff point was 0.15, which was equal to 3 times the mean OD of preimmune serum (*5*). Hyperimmune rabbit antiserum reacted strongly against the homologous VLPs (Table 2, Figure 3). SaV GII/3 Syd53 and GIV/1 Syd3 antisera titers were 512,000 and 2,056,000, respectively (Table 2). Several antisera weakly cross-reacted with heterologous VLPs. SaV GI/1 Mc114 antiserum cross-reacted weakly with GI/5 Yokote1 and GII/3 Syd53 VLPs, i.e., their cross-reactivities were 8- and 16-fold lower than that of the homologous VLP titer, respectively. SaV GI/5 Yokote1 antiserum cross-reacted weakly with GI/1 Mc114 and GII/3 Syd53 VLPs, i.e., its cross-reactivity was 16-fold lower than that of the homologous VLP titer. SaV GII/3 Syd53, GIV/1 Syd3, and GV/1 NK24 antisera appeared to have no cross-reactivities against any of the heterologous VLPs, i.e., their cross-reactivities were >32-fold lower than those of the homologous VLP titer. These results suggested that SaV GI/1 Mc114 and GI/5 Yokote1 antiserum had weak 2-way cross-reactivities against GI/5 Yokote1 and GI/1 Mc114 VLPs, respectively. The negative control NoV Osaka659 antiserum showed no cross-reactivities against VLPs of SaV at any dilution of antiserum, which indicates that the antiserum was specific for the VLPs and not the insect cell proteins.

Antigen ELISA Analysis

On the basis of a previous study, a specimen with an A_{492} (P – N) >0.10 and a P/N ratio >1.34 was considered significantly positive (*4*). Our recent antigen ELISA results showed that SaV GI Mc114, GI/5 Yokote1, and GV/1 NK24 VLPs were antigenically distinct (*19*), i.e., GI/1 Mc114 P– N 0.41, P/N 9.19; GI/5 Yokote1 P – N 0.93,

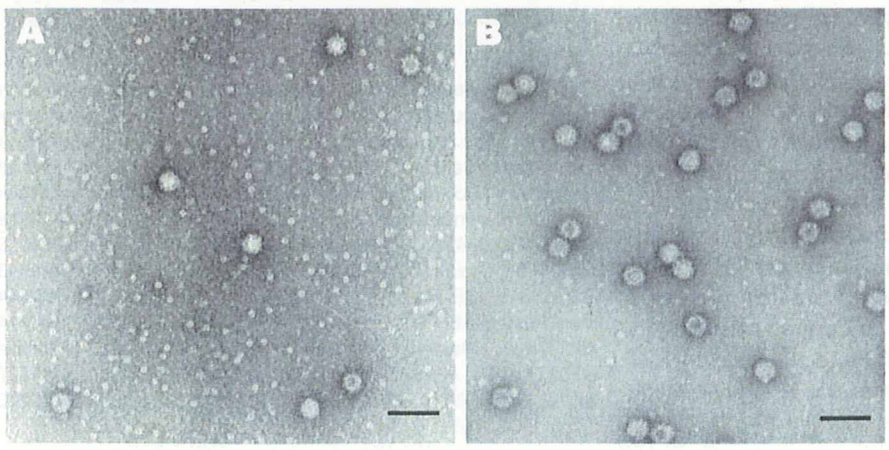

Figure 2. Electron micrographs of A) Syd53 and B) Syd3 viruslike particles of sapovirus. Scale bars = 100 nm.