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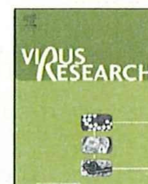

Short communication

Sapovirus-like particles derived from polyprotein

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ABSTRACT

We expressed full-length sapovirus genome constructs in insect cells and analyzed their products. The capsid protein was cleaved from the ORF1 polyprotein from a native-like genome construct and two full-length genome constructs with mutations in an active polymerase motif, whereas the capsid protein was not cleaved from a full-length genome construct with a mutation in an active protease motif. Our results showed that the sapovirus protease–polymerase precursor protein cleaved the capsid protein from the polyprotein at the putative conserved capsid start. Importantly, the cleaved capsid protein formed empty virus-like particles that were morphologically and antigenically similar to native sapovirus.

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The family *Caliciviridae* contains four genera *Sapovirus*, *Norovirus*, *Lagovirus*, and *Vesivirus*, which include *Sapporo virus*, *Norwalk virus*, *Rabbit hemorrhagic disease virus*, and *Feline calicivirus* species, respectively. Human sapovirus is an etiological agent of gastroenteritis and recent studies have found it to be an important cause of outbreaks of gastroenteritis (Chiba et al., 2000; Hansman et al., 2007a,c; Johansson et al., 2005; Noel et al., 1997). Sapovirus can be divided into five genogroups (GI to GV), of which GI, GII, GIV, and GV infect humans, while GIII infect porcine species (Farkas et al., 2004). The sapovirus genome is a single-stranded, positive sense RNA molecule of approximately 7.5 kb that is polyadenylated at the 3′ end. The sapovirus GI, GIV, and GV genomes are each predicted to contain three main open reading frames (ORFs), whereas sapovirus GII and GIII have two ORFs. Sapovirus ORF1 encodes for nonstructural proteins (including the VPg, protease, and RNA-dependent RNA polymerase) and a major capsid protein (VP1). Sapovirus ORF2 and ORF3 encode proteins of yet unknown functions. The predicted human sapovirus VP1 start contains an amino acid motif, MEG, which is conserved in all human sapovirus strains and as such is considered the putative VP1 start as well as near a putative subgenomic RNA start. Therefore, sapovirus virions may be formed from the genomic RNA and/or the subgenomic RNA. Human sapovirus is noncultivable, but expression of a subgenomic-like construct (i.e., VP1 to genome end) or VP1 alone in insect or mammalian cells can result in the formation of virus-like particles (VLPs) that are morphologically and antigenically similar to native sapovirus (Chen et al., 2004; Guo et al., 2001; Hansman et al., 2006a, 2007b; Jiang et al., 1999; Numata et al., 1997; Oka et al., 2006a). We recently determined the complete nucleotide sequence of a human sapovirus GII Mc10 strain (AY237420) and analyzed the proteolytic processing of the ORF1 polyprotein in an *in vitro* translation system and bacterial cells (Oka et al., 2005a,b, 2006b). The ORF1 polyprotein contained several amino acid motifs similar to the picornavirus, including the 3C-like protease and the 3D-like RNA-dependent RNA polymerase. We also defined the cleavage sites of Mc10 ORF1 polyprotein by expressing mutant constructs in an *in vitro* transcription–translation system and by N-terminal amino acid sequencing of the *Escherichia coli*-expressed proteins (Oka et al., 2006b), however evidence to form VLPs from the translated ORF1 polyprotein remains unclear. The purpose of the current study was to express different full-length sapovirus constructs in insect cells in order to better understand the proteolytic processing of human sapovirus capsid and the formation of VLPs.

Sapovirus GI Mc114 strain (GenBank accession number, AY237422) was isolated from a male infant seven months of age from the McCormic Hospital, Chiang Mai, Thailand on the 7 May 2001 (Hansman et al., 2004). A plasmid harboring the entire Mc114 genome was constructed in the same manner as sapovirus Mc10 strain (Oka et al., 2005a), except for different sets of primers (data not shown). The full-length Mc114 plasmid was used as a template for designing four full-length constructs (Fig. 1). One construct was analogous to the full-length native sequence (termed Native construct); one construct contained a mutation in the protease gene (GDAG construct); and two constructs

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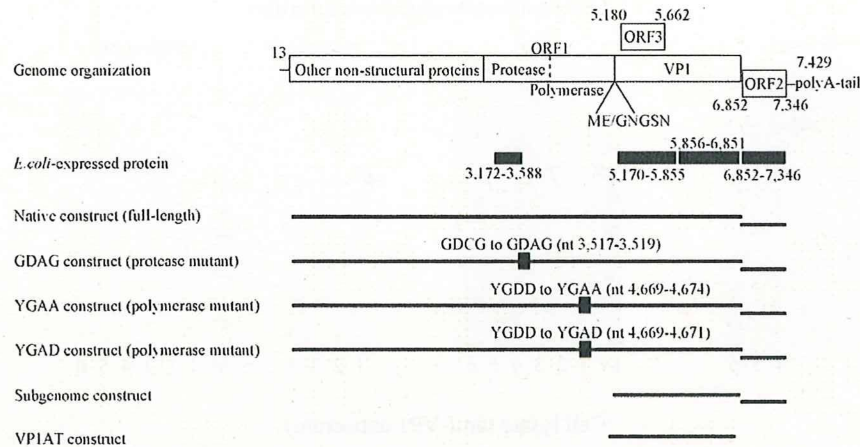

Fig. 1. The predicted genomic organization of sapovirus Mc114 strain was based on sapovirus Mc10 cleavage experiments (Oka et al., 2005b). The six different constructs expressed in the insect cells and the relative positions of the mutations in the protease and polymerase genes are shown (asterisks). The Native construct was identical to the native genome. The protease mutant construct was identical to the Native construct, except with a defective protease gene, while the polymerase mutant (YGAA and YGAD) constructs had defective polymerase genes. The Subgenome construct was analogous to the subgenome and expressed VP1 and ORF2, but not nonstructural proteins. The VP1AT construct only expressed the VP1 protein.

contained mutations in the polymerase gene (YGAA and YGAD constructs). In order to design the mutant constructs, we used the Gene Tailor site-directed mutagenesis kit (Invitrogen). Briefly, for the protease mutant, we disrupted the GDCG motif, converting nucleotides TGT (^{1,169}Cys) to GCG (^{1,169}Ala, italics) using sense primer (5′-ACTAAGAAGGGAGAC*GCG*GGACTGCCCTATTTCAAC-3′) and antisense primer (5′-GTCTCCCTTCTTAGTTGGGTAACCATTCAC-3′). For the polymerase mutants, we disrupted the YGDD motif. For the YGAA construct, we converted nucleotides GATGAC (^{1,553}Asp^{1,554}Asp) to GCTGCC (^{1,553}Ala^{1,554}Ala, italics) using sense primer (5′-CCATCCACACGTACGGT*GCTGCC*TGCATGTACAGTGTGTG-C-3′) and antisense primer (5′-CACCGTACGTGTGGATGGTCTCCACC-TGGAAG-3′). For the YGAD construct, we converted nucleotides GAT (^{1,553}Asp) to GCC (^{1,553}Ala, italics) using sense primer (5′-CCATCCACACGTACGGT*GCC*GACTGCATGTACAGTGTGTGC-3′) and antisense primer (5′-CACCGTACGTGTGGAT GGTCTCCACCTGGAAG-3′). The full-length constructs were finally amplified using a sense primer that was designed to start at the genome start (italics) (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC-*GTGATTGGTTAGATGGCTTCCAAG*-3′) and an antisense primer at the genome end TX30SXNattb2 (5′-GACTAGTTCTAGATCGCGAGCGGCC-GCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′). As a control, we used a subgenomic-like construct (termed Subgenome construct) (Hansman et al., 2005) and a newly designed construct that contained only the VP1 gene (termed VP1AT). For the VP1AT construct, we used the Subgenome construct sense primer (Hansman et al., 2005) and an antisense primer (5′-GGGGACCACTTTGTACAAGAA-AGCTGGGTCTCATTGGAACACCCGTTTGGCCCTTC-3′). PCR-amplified fragments were cloned into the Gateway Expression System (Invitrogen, Carlsbad, Calif.) and then expressed in insect cells as previously described (Hansman et al., 2005, 2006b). Antisera were raised against an *E. coli*-expressed Mc114 protease protein (corresponding to the nucleotide region 3172–3588) using a method previously described (Oka et al., 2005b), as well as an Mc114 N-terminal-half-VP1 (corresponding to the nucleotide region 5170–5855), a Mc114 C-terminal-half-VP1 (corresponding to the nucleotide region 5856–6851), and an entire Mc114 ORF2 protein (corresponding to the nucleotide region 6852–7346) (Hansman et al., 2005). We also used antiserum raised against purified insect cell expressed Mc114 VLPs (Hansman et al., 2005).

The Mc114 genome was determined to be 7429 nucleotides (nt), excluding the poly(A) tail, and was predicted to encode three main ORFs (Fig. 1). The ORF1 was 6840 nt (nt 13–6852) and encoded a polyprotein of 251 kDa. The ORF2 was 495 nt (nt 6852–7347) and encoded a protein of 17 kDa. The ORF3 was 483 nt (nt 5180–5662), which overlapped the VP1 gene by 11 nt, and encoded a protein of 17 kDa.

Western blotting and electron microscopy (EM) were used to examine the time-course expression of six different Mc114 constructs (Fig. 1) in insect cells. The cell lysate and culture medium were collected at 1, 2, 3, 4, 5, and 6 days post infection (dpi) as previously described (Hansman et al., 2005, 2006b). The cell lysate was analyzed using anti-protease and anti-VLP antisera, whereas the culture medium was analyzed using anti-VLP, anti-N-terminal-half-VP1, anti-C-terminal-half-VP1, and anti-ORF2 antisera.

A 70-kDa band was detected in the Native, YGAA, and YGAD cell lysates at 2 dpi using anti-protease antiserum (Fig. 2A). The 70-kDa band likely represented a protease–polymerase precursor protein, since it was not detected in the GDAG, Subgenome, or VP1AT cell lysates using anti-protease antiserum (Fig. 2A). The protease–polymerase protein was not cleaved further, suggesting that the protease–polymerase protein was a stable product in the insect cells. Several other calicivirus studies also identified the protease–polymerase protein as a stable product (Belliot et al., 2003; Martin Alonso et al., 1996; Oka et al., 2005b; Sosnovtseva et al., 1999). A band of approximately 250-kDa was detected in the GDAG cell lysate between 2 and 6 dpi using anti-protease antiserum (Fig. 2A) and between 2 and 5 dpi using anti-VP1 antiserum (Fig. 2B). The 250-kDa band likely represented the uncleaved ORF1 polyprotein, since it was not detected with the other constructs. Taken together, these results show that the ORF1 polyprotein was expressed in insect cells and the mutation in the protease gene (GDAG construct) disrupted the proteolytic processing of the ORF1 polyprotein. Interestingly, the protease activity was not affected by the mutations in the polymerase gene (YGAA and YGAD constructs), indicating that the sapovirus polymerase activity was not required for proteolytic processing in insect cells. Also, the GDAG construct appeared less stable than the other constructs as numerous additional other bands were detected (Fig. 2A and B). A similar result using a protease mutant construct was also observed in a rabbit hemorrhagic disease virus expression study (Sibilia et al., 1995).

A 60-kDa band was detected in the YGAA, YGAD, and Subgenome cell lysates from 2 dpi and in the VP1AT cell lysate at 5 dpi using anti-VLP antiserum (Fig. 2B). The 60-kDa band was detected in the

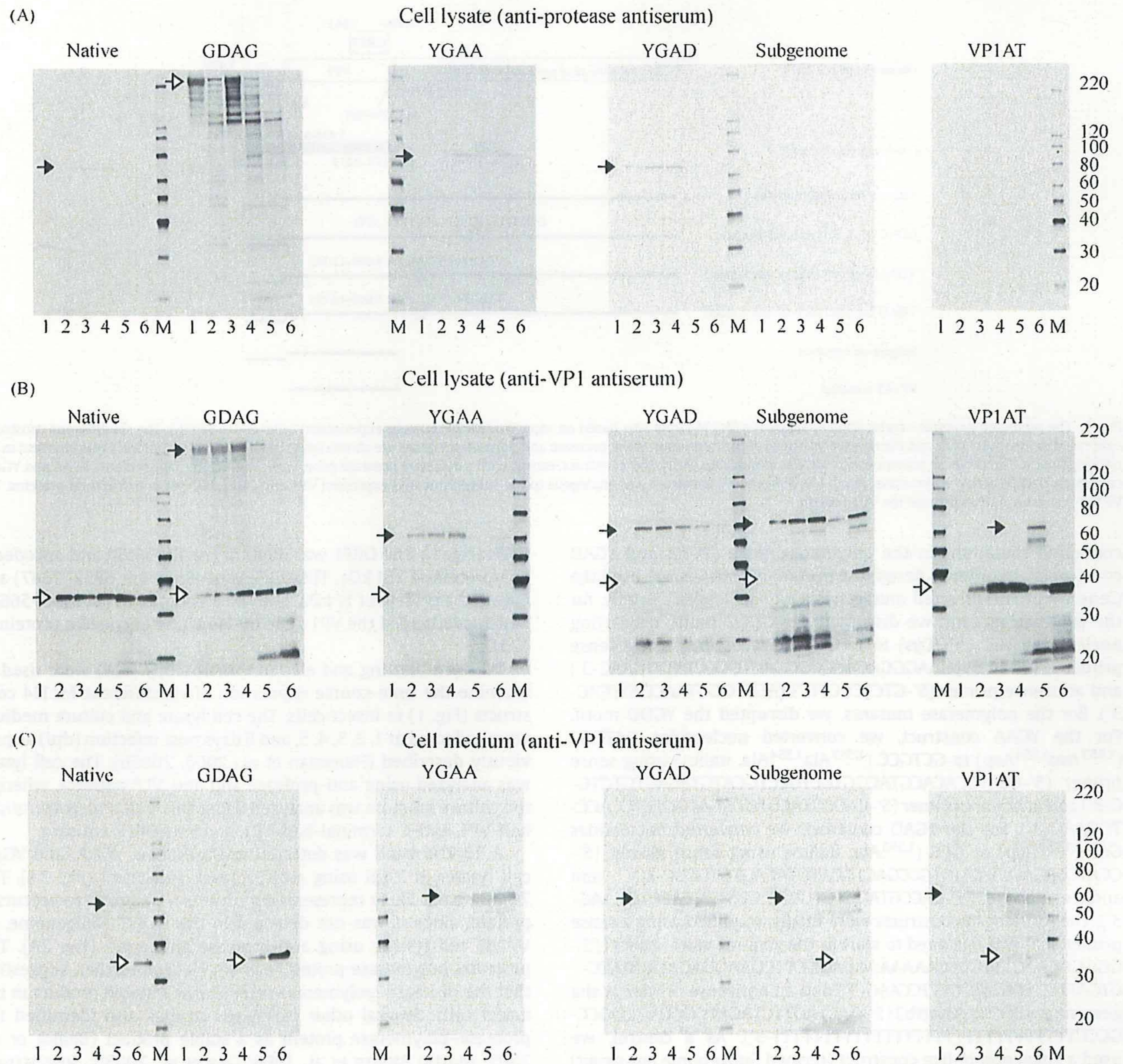

Fig. 2. A time-course expression of the six different sapovirus constructs in insect cells. The cell lysate and supernatant were collected at 1, 2, 3, 4, 5, and 6 dpi and examined by Western blotting (M = protein marker). (A) Cell lysate proteins were detected using the anti-protease antiserum (A), where the black arrows showed the protease–polymerase protein of 70 kDa and the white arrow showed the uncleaved ORF1 polyprotein of approximately 250 kDa. Cell lysate (B) and culture medium (C) proteins were detected using the anti-VLP antiserum, where the black arrows showed the VP1 proteins of approximately 60 kDa and the white arrows showed the C-terminal truncated ORF1 protein of approximately 36 kDa.

Native, YGAA, YGAD, Subgenome, and VP1AT culture medium at 4 or 5 dpi using anti-VLP antiserum (Fig. 2C). The 60-kDa band represented the VP1, as previously identified (Hansman et al., 2005). VP1 was not detected in the GDAG cell lysate or culture medium (Fig. 2B and C), which clearly shows that the proteolytic processing of the ORF1 polyprotein was performed by the sapovirus protease–polymerase protein and not the cellular and/or baculovirus proteases. In addition, our results show that the sapovirus polymerase activity was not required for proteolytic processing of VP1, i.e., VP1 was detected with the two polymerase mutant constructs (YGAA and YGAD). However, a band of approximately 36-kDa was detected in the cell lysate and/or culture medium with all of the constructs (Fig. 2B and C). We were able to detect the 36-kDa band using anti-C-terminal-VP1 antiserum, but not with the anti-N-terminal-VP1 antiserum (data not shown), which suggests that the C-terminal-half of VP1 for all the constructs was cleaved by cellular and/or baculovirus proteases.

The cell lysate and culture medium was examined for ORF2 expression, however no bands were detected (see Supplementary Fig. 1). These results suggest that sapovirus ORF2 was not expressed in insect cells or the level was too low. In a similar study, norovirus VP2 (analogous to sapovirus ORF2) was found to stabilize VP1 expression and protect VP1 from protease degradation, although the level of VP2 expression was found to be low (Bertolotti-Ciarlet

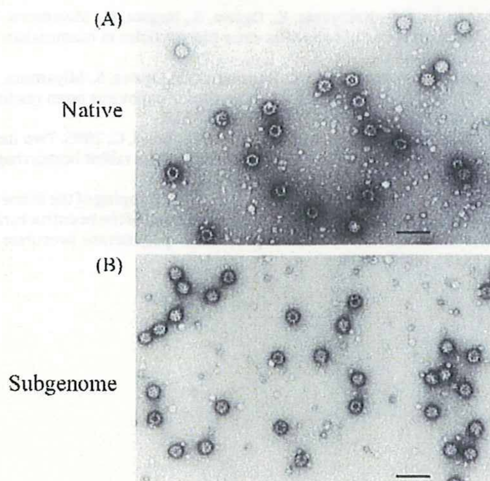

Fig. 3. Electron microscopy images of the VLPs expressed in insect cells for the (A) full-length Native construct and (B) Subgenome construct at 6 dpi. The bar represents 100 nm.

et al., 2003). Interestingly, sapovirus VLPs could still be formed without the ORF2 sequence (VP1AT construct). Further studies are needed to explain the function(s) of sapovirus ORF2 and whether or not it stabilizes VP1 expression and prevents VP1 degradation.

We examined the 4, 5, and 6 dpi culture medium for the formation of VLPs using EM. We found VLPs 41–46 nm in diameter in the Native, YGAA, YGAD, Subgenome, and VP1AT culture medium at 5 or 6 dpi (Fig. 3). VLPs were not detected in the GDAG culture medium or cell lysate. In order to determine density of the VLPs, the Native and Subgenome constructs were each infected in confluent Tn5 cells in a total of 300 ml of Ex-Cell 405 medium followed by incubation at 26 °C for 5 days. The VLPs were harvested from the culture medium and then CsCl equilibrium gradient ultracentrifugation was performed as previously described (Hansman et al., 2005). Approximately 200 μl per fraction was collected from the bottom of the tube, the density measured, and then each fraction was analyzed using an antigen ELISA (Hansman et al., 2005) and EM. The greatest OD value was observed in fractions 15 and 16 for both constructs, which corresponded to approximately 1.27 g/ml (data not shown). Similarly, a major peak at the same density was observed with the YGAA and YGAD constructs (data not shown). We found that the greatest numbers of VLPs were in fractions 15 and 16, however VLPs were sparsely distributed in other densities, indicating a tailing or carry-over during the CsCl method. Nevertheless, these results show that the sapovirus RNA (neither genomic nor subgenomic) was not packaged inside the VLPs and a similar density was reported for a feline calicivirus study (Geissler et al., 1999).

To further investigate the cleaved VP1 proteins, protein sequence was determined for Native, YGAA, and YGAD constructs using an Edman's degradation method (APRO Science, Japan). We were unable to determine the N-terminal protein sequence for the Subgenome VP1. The VP1 N-terminal amino acid sequence for the Native, YGAA, and YGAD was found to be GNGSN. These results show that the sapovirus protease–polymerase cleaved the VP1 from the polyprotein between ^{1721}E and ^{1722}G. This site is highly conserved among all human sapoviruses (Hansman et al., 2005; Oka et al., 2006a) and provides strong evidence that the putative VP1 start motif, i.e., MEG, was in fact the genuine human sapovirus VP1 start.

In conclusion, our results have shown for the first time that the sapovirus 70-kDa protease–polymerase precursor protein was indeed active in insect cells. We found that the sapovirus protease–polymerase protein cleaved the VP1 from the ORF1 polyprotein and then the cleaved VP1 successfully folded into VLPs. This new finding suggests that human sapovirus virions could be assembled from the ORF1 and/or the subgenomic RNA. We also showed that the sapovirus polymerase activity was not required for capsid protein processing. The ability to produce "near native-like" VLPs may contribute to the development of effective diagnostic reagents and vaccines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2008.07.002.

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Research Note

Detection of Human Enteric Viruses in Japanese Clams

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ABSTRACT

A total of 57 clam packages that were collected from supermarkets and fish markets from 11 different sites in western Japan between 8 December 2005 and 6 September 2006 were examined for human enteric viruses (i.e., norovirus, Aichi virus, rotavirus, adenovirus, hepatitis A virus, and astrovirus), using PCR and reverse transcription PCR. Sixty-one percent of the packages were contaminated with one type of virus, 9% had two different types of viruses, 28% had three different types of viruses, and 9% had at least four different types of viruses. Thirty-one (54%) of 57 packages were contaminated with noroviruses. Norovirus genogroup I and genogroup II sequences were detected in 24 and 23 packages, respectively, and these sequences belonged to nine genogroup I and eight genogroup II genotypes. Aichi viruses were found in 19 (33%) of 57 packages, and these belonged to genogroup A. Rotaviruses (group A) were detected in 14 (42%) of 33 of packages and 9 of 14 rotavirus-positive packages contained two or more rotavirus genogroup types. Adenoviruses (Ad40 and Ad41) were detected in 17 (52%) of 33 packages. One of the 57 (2%) packages was positive with hepatitis A virus (subtype IA). Astrovirus was not detected in any of the packages. This is the first study to detect such a high level of contamination in Japanese clams. These results represent an important finding because the Japanese clams were considered suitable for human consumption. Further studies are needed to determine the health risks associated with eating these highly contaminated clams.

Gastroenteritis is one of the leading causes of death by an infectious disease *(19)*, with more than 700 million cases of acute diarrheal disease occurring annually. The main viral agents that cause gastroenteritis are norovirus, rotavirus, sapovirus, astrovirus, and enteric adenoviruses. These viruses have been detected in environmental samples (e.g., lakes and sewage) as well as in foods such as oysters, clams, sandwiches, and raspberries. Other important viral agents that can accumulate in oysters and clams are hepatitis A virus (HAV) and hepatitis E virus (HEV) *(4, 17)*. The impact of viral contamination in the environment is evident in Japan, where outbreaks of norovirus oyster–associated gastroenteritis increases in winter, and this coincides with the oyster-harvesting season in winter *(21)*. The detection methods for these viruses in environmental samples and clinical specimens have greatly improved over the past 10 years and have provided a better understanding and distribution of these viruses.

The purpose of this study was to detect norovirus, Aichi virus, rotavirus, adenovirus, HAV, and astrovirus in clam packages sold at supermarkets and fish markets, which were destined for human consumption in Japan, using PCR and reverse transcription PCR, and then describe the genetic diversity of the positive noroviruses.

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MATERALS AND METHODS

Clam samples. A total of 57 clam (*Corbicula japonica*) packages (30 to 60 clams per package) were collected from supermarkets or fish markets (nonexport) from 11 different geographically distinct sites in western Japan between 8 December 2005 and 6 September 2006. The clam packages were screened for norovirus, Aichi virus, rotavirus, adenovirus, HAV, and astrovirus using PCR and reverse transcription PCR. These 57 packages were previously screened for sapovirus *(9)*, and 46 of 57 packages were screened for HEV *(17)*.

Viral extraction. The clams were shucked, the digestive diverticulum removed by dissection on the day of harvest *(16)*, and then weighed and homogenized in nine times their weight of phosphate-buffered saline without magnesium or calcium. One gram of digestive diverticulum (10 to 15 clams per package) was homogenized with an Omni mixer (Omni International, Marietta, Ga.) in 10 ml of phosphate-buffered saline (pH 7.2). After centrifugation at 10,000 × *g* for 30 min at 4°C, the supernatant was layered onto 1 ml of 30% sucrose solution and ultracentrifuged at 154,000 × *g* for 3 h at 4°C. The pellet was resuspended in 140 μl of distilled water and stored at −80°C until use.

DNA and RNA extraction and reverse transcription. Viral DNA (for detection of adenovirus) was extracted from resuspended pellet, using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Viral RNA (for detection of norovirus, Aichi virus, rotavirus, and HAV) was extracted from the resuspended pellet, using QIAmp Viral RNA Mini Kit (Qiagen,). For reverse transcription, the RNA solution was treated with 2 U of RNase-free DNase I (Takara, Tokyo, Japan) for 30 min at 37°C, and was

TABLE 1. *Details of the clam samples*

Package no.	Site	Mo/day/yr	No. of norovirus genotypes	Norovirus strain name (genogroup/genotype)	Aichi virus	Rotavirus: G types	Adeno-virus	HAV	HEV (from *(17)*)	Sapovirus (from *(9)*)
1	A	12/08/05	2	GI-Shijimi1 (GI/4), GII-Shijimi1 (GII/3)	Aic-1	G1/G4	–	–	–	–
2	D	12/10/05	–		–	–	+	–	–	–
3	A	12/22/05	2	GI-Shijimi2 (GI/1), GII-Shijimi2 (GII/2)	Aic-2	G9	+	–	–	–
4	D	12/17/05	–		–	G8	+	–	–	–
5	K	01/10/06	1	GII-Shijimi3 (GII/3)	Aic-3	NTa	NT	–	–	–
6	A	01/14/06	3	GI-Shijimi4 (GI/11), GII-Shijimi4a (GII/4), GII-Shijimi4b (GII/3)	Aic-4	G4	+	–	–	–
7	J	01/16/06	1	GI-Shijimi5 (GI/8)	Aic-5	NT	NT	–	–	–
8	A	01/20/06	2	GI-Shijimi6 (GI/14), GII-Shijimi6 (GII/3)	–	NT	NT	–	–	–
9	B	01/22/06	3	GI-Shijimi7 (GI/12), GII-Shijimi7a (GII/New), GII-Shijimi7b (GII/3)	Aic-6	G?	+	–	–	–
10	B	01/22/06	–		–	–	+	–	–	–
11	C	01/21/06	3	GI-Shijimi8 (GI/11), GII-Shijimi8a (GII/3), GII-Shijimi8b (GII/3), GII-Shijimi8c (GII/4), GII-Shijimi8d (GII/3)	–	–	–	–	–	–
12	D	01/24/06	3	GI-Shijimi9 (GI/8), GII-Shijimi9a (GII/3), GII-Shijimi9b (GII/4)	Aic-7	G?	+	–	–	–
13	C	01/26/06	3	GII-Shijimi10a (GII/3), GII-Shijimi10b (GII/3), GII-Shijimi10c (GII/3)	Aic-8	–	+	–	–	SaV-3
14	D	02/07/06	1	GI-Shijimi11 (GI/11)	Aic-18	NT	NT	–	+	–
15	B	02/05/06	4	GI-Shijimi12a (GI/1), GI-Shijimi12b (GI/1), GI-Shijimi12c (GI/8), GI-Shijimi12d (GI/11)	Aic-9	–	+	–	–	–
16	D	02/19/06	2	GI-Shijimi13 (GI/1), GII-Shijimi13 (GII/3)	Aic-10	G1/G8/G9	+	–	–	–
17	B	02/17/06	1	GII-Shijimi14 (GII/New)	–	G4	+	–	–	–
18	C	02/25/06	1	GII-Shijimi15 (GII/3)	Aic-11	G2/G4	–	–	–	–
19	D	03/01/06	7	GI-Shijimi16a (GI/11), GI-Shijimi16b (GI/8), GI-Shijimi16c (GI/4), GI-Shijimi16d (GI/8), GII-Shijimi16a (GII/3), GII-Shijimi16b (GII/4), GII-Shijimi16c (GII/4)	Aic-12	NT	NT	–	+	–
20	B	03/02/06	3	GI-Shijimi17 (GI/1), GII-Shijimi17a (GII/5), GII-Shijimi17b (GII/4)	Aic-19	–	+	–	–	–
21	C	03/10/06	2	GI-Shijimi18 (GI/8), GII-Shijimi18 (GII/3)	–	–	+	–	–	SaV-1
22	B	03/14/06	3	GI-Shijimi19a (GI/1), GI-Shijimi19b (GI/1), GII-Shijimi19 (GII/3)	Aic-13	–	+	–	–	–
23	A	03/14/06	–		–	NT	NT	–	–	–
24	E	03/13/06	–		–	NT	NT	–	–	–
25	E	03/14/06	–		–	NT	NT	–	–	–
26	B	03/15/06	2	GI-Shijimi20a (GI/5), GI-Shijimi20b (GI/14)	Aic-14	–	+	–	–	–
27	D	03/17/06	1	GI-Shijimi21 (GI/1)	–	G1/G2/G8	+	–	–	–
28	F	03/18/06	2	GI-Shijimi22 (GI/8), GII-Shijimi22 (GII/New)	–	G1/G2/G3/G8/G9	+	–	–	–
29	E	03/18/06	–		–	NT	NT	–	–	–
30	E	03/18/06	–		–	NT	NT	–	–	–

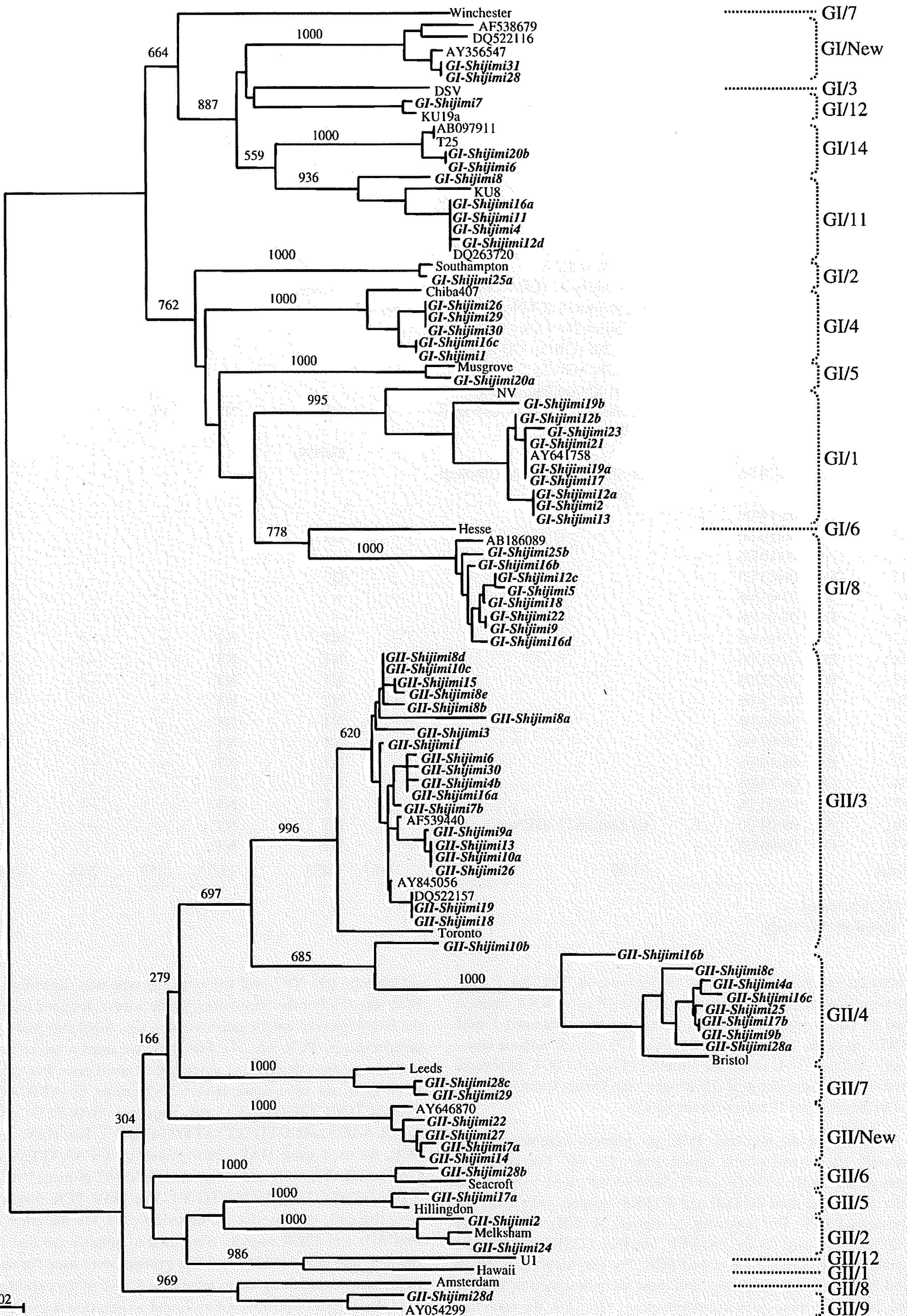
TABLE 1. *Continued*

Package no.	Site	Mo/day/yr	No. of norovirus genotypes	Norovirus strain name (genogroup/genotype)	Aichi virus	Rotavirus: G types	Adeno-virus	HAV	HEV (from *(17)*)	Sapovirus (from *(9)*)
31	G	03/18/06	1	GI-Shijimi23 (GI/1)	–	NT	NT	–	–	–
32	H	03/18/06	–		–	NT	NT	–	–	–
33	D	03/30/06	1	GII-Shijimi24 (GII/2)	Aic-15	G1/G8	–	–	–	–
34	B	04/07/06	3	GI-Shijimi25a (GI/2), GI-Shijimi25b (GI/8), GII-Shijimi25 (GII/4)	–	–	–	–	–	–
35	D	04/13/06	2	GI-Shijimi26 (GI/4), GII-Shijimi26 (GII/3)	Aic-16	G1/G2/G8	–	–	–	SaV-2
36	B	04/26/06	1	GII-Shijimi27 (GII/New)	–	G2/G4	–	–	–	–
37	A	05/16/06	5	GI-Shijimi28 (GI/New), GII-Shijimi28a (GII/4), GII-Shijimi28b (GII/6), GII-Shijimi28c (GII/7), GII-Shijimi28d (GII/9)	Aic-17	–	–	–	–	SaV-4
38	D	05/13/06	2	GI-Shijimi29 (GI/4), GII-Shijimi29 (GII/7)	–	NT	NT	1A	–	–
39	D	05/27/06	–		–	G8/G9	–	–	–	–
40	A	05/29/06	2	GI-Shijimi30 (GI/4), GII-Shijimi30 (GII/3)	–	–	–	–	–	–
41	I	06/14/06	–		–	–	–	–	–	–
42	D	06/16/06	–		–	G3	–	–	–	–
43	A	06/16/06	–		–	–	–	–	–	–
44	D	06/23/06	–		–	G?	–	–	–	–
45	B	07/05/06	–		–	–	–	–	–	–
46	D	07/06/06	–		–	–	–	–	–	–
47	A	07/13/06	–		–	NT	NT	–	–[b]	–
48	D	07/21/06	–		–	NT	NT	–	–[b]	–
49	B	07/21/06	–		–	NT	NT	–	–[b]	–
50	A	07/27/06	–		–	NT	NT	–	–[b]	–
51	A	08/06/06	–		–	NT	NT	–	–[b]	–
52	D	08/07/06	–		–	NT	NT	–	–[b]	–
53	B	08/10/06	–		–	NT	NT	–	–[b]	–
54	D	08/23/06	–		–	NT	NT	–	–[b]	–
55	I	09/04/06	–		–	NT	NT	–	–[b]	–
56	B	09/04/06	1	GI-Shijimi31 (GI/New)	–	NT	NT	–	–[b]	–
57	D	09/06/06	–		–	NT	NT	–	–[b]	–
Total				31/57	19/57	17/33	17/33	1/57	2/46	4/57

[a] NT, not tested.
[b] Tested in this study.

followed by the inactivation of the enzyme at 75°C for 5 min. Reverse transcription was performed with 15 μl of RNA solution and 15 μl of reverse transcription mixture that contained 1 mM dNTP mixture, 10 mM dithiothreitol, 0.75 μg of random hexamers (Takara), 33 U of RNase inhibitor (Takara), 300 U of reverse transcriptase Superscript II (Invitrogen, San Diego, Calif.), and 4.5 μl of Superscript II buffer (Invitrogen).

PCR. For the norovirus PCR, the primers were designed to amplify the 5′ end of the capsid gene *(10, 14)*. For norovirus genogroup I (GI), COG1F and G1SKR primers were used for the first PCR, and then G1SKF and G1SKR primers were used for the nested PCR. For norovirus genogroup II (GII), COG2F and G2SKR were used for the first PCR, and then G2SKF and G2SKR primers were used for the nested PCR. For the Aichi virus, C94b and 264K primers were used, and these were designed to amplify the 3C–D junction (protease-polymerase) *(26)*. For the rotavirus (group A), primers were designed to amplify the major outer capsid glycoprotein VP7, and the rotavirus type was determined by PCR size *(7)*. For the adenovirus, primers were designed to detect the E1B region of enteric adenoviruses, i.e., Ad40 and Ad41, and determined by PCR size *(1)*. For HAV, we used a set of nested in-house primers designed to amplify the capsid gene. For the first HAV PCR, we used sense HAV+2799 primer (5′-ATTCAGAT TAGACTGCCTTGGTA-3′) and antisense HAV-3273 primer (5′-CCAAGAAACCTTCATTATTTCATG-3′). For HAV nested PCR, we used sense HAV+2907 primer (5′-GCAAATTACAAT CATTCTGATGA-3′) and antisense HAV-3162 primer (5′-CTTC YTGAGCATACTTKARTCTTG-3′). The HAV PCR conditions were the same as those for the norovirus *(14)*. For the astrovirus, PreCAP1 and 12GR primers were used to amplify the first PCR product, and then Mon244 and 82b primers were used for nested PCR, which were designed to amply the 5′ end of the capsid gene *(18)*. Two types of positive controls and a virus-free negative control per five assays for norovirus PCR were used. All PCR prod-


Winchester
AF538679
DQ522116
AY356547
GI-Shijimi31
GI-Shijimi28
DSV
GI-Shijimi7
KU19a
AB097911
T25
GI-Shijimi20b
GI-Shijimi6
GI-Shijimi8
KU8
GI-Shijimi16a
GI-Shijimi11
GI-Shijimi4
GI-Shijimi12d
DQ263720
Southampton
GI-Shijimi25a
Chiba407
GI-Shijimi26
GI-Shijimi29
GI-Shijimi30
GI-Shijimi16c
GI-Shijimi1
Musgrove
GI-Shijimi20a
NV
GI-Shijimi19b
GI-Shijimi12b
GI-Shijimi23
GI-Shijimi21
AY641758
GI-Shijimi19a
GI-Shijimi17
GI-Shijimi12a
GI-Shijimi2
GI-Shijimi13
Hesse
AB186089
GI-Shijimi25b
GI-Shijimi16b
GI-Shijimi12c
GI-Shijimi5
GI-Shijimi18
GI-Shijimi22
GI-Shijimi9
GI-Shijimi16d
GII-Shijimi8d
GII-Shijimi10c
GII-Shijimi15
GII-Shijimi8e
GII-Shijimi8b
GII-Shijimi8a
GII-Shijimi3
GII-Shijimi1
GII-Shijimi6
GII-Shijimi30
GII-Shijimi4b
GII-Shijimi16a
GII-Shijimi7b
AF539440
GII-Shijimi9a
GII-Shijimi13
GII-Shijimi10a
GII-Shijimi26
AY845056
DQ522157
GII-Shijimi19
GII-Shijimi18
Toronto
GII-Shijimi10b
GII-Shijimi16b
GII-Shijimi8c
GII-Shijimi4a
GII-Shijimi16c
GII-Shijimi25
GII-Shijimi17b
GII-Shijimi9b
GII-Shijimi28a
Bristol
Leeds
GII-Shijimi28c
GII-Shijimi29
AY646870
GII-Shijimi22
GII-Shijimi27
GII-Shijimi7a
GII-Shijimi14
GII-Shijimi28b
Seacroft
GII-Shijimi17a
Hillingdon
GII-Shijimi2
Melksham
GII-Shijimi24
U1
Hawaii
Amsterdam
GII-Shijimi28d
AY054299
664
887
1000
559
1000
936
1000
762
1000
1000
995
778
1000
620
996
697
685
1000
279
166
1000
1000
304
1000
1000
1000
986
969
0.02
GI/7
GI/New
GI/3
GI/12
GI/14
GI/11
GI/2
GI/4
GI/5
GI/1
GI/6
GI/8
GII/3
GII/4
GII/7
GII/New
GII/6
GII/5
GII/2
GII/12
GII/1
GII/8
GII/9

ucts were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Norovirus, Aichi virus, and HAV nucleotide sequences were prepared as previously described *(11)*.

Sequence analysis. Norovirus, Aichi virus and HAV nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1, Applied Biosystems, Warrington, UK) and determined with the ABI 3130 sequencer (ABI, Boston, Mass.). In order to determine the norovirus genotypes in the packages with multiple genotypes, we cloned the reverse transcription PCR products into pCR2.1 (Invitrogen), and at least four clones from each sample were sequenced. The genetic diversity of the adenoviruses was not determined in this study. Norovirus nucleotide sequences were aligned with ClustalX, and the distances were calculated by Kimura's two-parameter method. The norovirus nucleotide sequence data determined in this study has been deposited in GenBank under accession no. EF424485 through EF424557.

RESULTS

Thirty-five (61%) of 57 packages were contaminated with one type of virus, 5 (9%) of 57 packages were contaminated with two different types of viruses, 16 (28%) of 57 packages were contaminated with three different types of viruses, and 5 (9%) of 57 packages were contaminated with at least four different types of viruses (Table 1). Astrovirus was not detected in any of the packages.

Noroviruses. Thirty-one (54%) of 57 packages were contaminated with noroviruses (Table 1). Norovirus GI and GII sequences were detected in 24 and 23 packages, respectively (Fig. 1). A total of 24 norovirus GI sequences were detected, and these clustered into nine different GI genotypes (Fig. 1), including one unpublished GI genotype (GI/1, GI/2, GI/4, GI/5, GI/8, GI/11, GI/12, GI/14, and GI/New). A total of 23 norovirus GII sequences were detected, and these clustered into eight different GII genotypes (Fig. 1), including one unpublished GII genotype (GII/2, GII/3, GII/4, GII/5, GII/6, GII/7, GII/9, and GII/New). More than half of the norovirus-positive packages, 20 (65%) of 31, contained two or more norovirus genotypes. Twenty-three (74%) of 31 norovirus-positive packages were co-contaminated with two or more other types of viruses (Table 1).

Aichi virus. We found that 19 (33%) of 57 packages were contaminated with Aichi viruses. The 19 Aichi virus sequences shared over 95% nucleotide homology, suggesting that the same strain contaminated the clams. These 19 sequences closely matched (approximately 95% nucleotide homology) genogroup A sequences found on the database (data not shown). All of the Aichi virus–positive packages were co-contaminated with other viruses (Table 1).

Rotavirus. Fourteen (42%) of 33 packages were contaminated with rotavirus (24 packages were unavailable for screening). Six different rotavirus G types were detected, i.e., G1, G2, G3, G4, G8, and G9. Of the 14 rotavirus-positive packages nine (53%) contained two or more rotavirus G types (Table 1).

Adenovirus. Seventeen (52%) of 33 packages were contaminated with adenoviruses, using primers designed to detect the two enteric adenoviruses, i.e., Ad40 and Ad41. Fourteen (82%) of 17 adenovirus-positive packages were co-contaminated with other viruses (Table 1).

HAV and HEV. One (2%) of 57 packages was contaminated with HAV. Sequence analysis of the capsid gene indicated that it belonged to subtype IA. HEV was previously detected in 2 of 46 packages *(17)*. An additional 11 packages were screened for HEV; however these were all negative (Table 1).

DISCUSSION

The current study has shown that Japanese clams (*C. japonica*) purchased in supermarkets and fish markets were highly contaminated with human enteric viruses from the natural environment. Similarly, a 3-year study in France found that mussel samples (*Mytilus galloprovincialis*) were highly contaminated with enteric viruses *(15)*. However, an important difference between the study conducted in France and the current study was that the French mussels were collected in areas where sewage was discharged and were prohibited for human consumption, whereas the Japanese clams were sold in supermarkets and fish markets and were considered suitable for human consumption.

Noroviruses are the dominant cause of outbreaks of gastroenteritis worldwide. In this study, the noroviruses were the dominant virus detected the clam packages (found in 54% of the packages). In a comparative study, noroviruses were detected in only approximately 5 to 9% of Japanese oysters (*Crassostrea gigas* or *Crassostrea nippona*) *(20, 21)*. These results suggested that the Japanese clams were more highly contaminated with noroviruses than were the Japanese oysters, or alternatively, it was just a reflection on the different collection sites, i.e., the clams were collected from brackish waters, whereas the oysters were collected from the sea. Alternatively, the different detection rates in clams and oysters were a result of the different sample preparations. Nevertheless, all of the norovirus sequences detected in the clam packages closely matched other sequences detected in patients with gastroenteritis in Japan (using GenBank BLAST searches), suggesting that the contaminated Japanese clams could cause gastroenteritis in humans, although direct evidence is lacking.

Over the past 10 years, the norovirus GII/4 strains have become the dominant cause of outbreaks of gastroenteritis

←

FIGURE 1. *Phylogenetic analysis of norovirus capsid sequences (approximately 300 nucleotides) showing the different genogroups and genotypes. The numbers on each branch indicate the bootstrap values for the genotype. Bootstrap values of 950 or higher were considered statistically significant for the grouping. The scale represents nucleotide substitutions per site. The frequency of each norovirus genotype was 9, 1, 5, 1, 8, 5, 1, 2, 2, 1, 20, 8, 1, 1, 2, 1, and 4 for GI/1, GI/2, GI/4, GI/5, GI/8, GI/11, GI/12, GI/14, GI/New, GII/2, GII/3, GII/4, GII/5, GII/6, GII/7, GII/9, and GII/New, respectively.*

worldwide. In a recent study, we also found that the GII/4 strains were the dominant cause of outbreaks of gastroenteritis in food-catering settings in Japan *(22)*. In the current study, the norovirus GII/3 sequences were detected more frequently than were the norovirus GII/4 sequences, i.e., 20 versus 8 sequences, respectively (Fig. 1). This result may only reflect that the GII/3 strains were more dominant in this area of Japan; however, the norovirus GII/3 strains were the second most dominant cause of gastroenteritis in Japan, Australia, and Vietnam *(2, 8, 22)*, indicating that this genotype is indeed a major cause of gastroenteritis. Noteworthy were two new norovirus genotypes (GI/New and GII/New; Fig. 1) detected in the clam packages, at three different sites, and several months apart. Similar norovirus sequences were recently reported in patients in Thailand, Taiwan, Hong Kong, and from an outbreak on a U.S. navy ship (data not shown), indicating that there may be a widespread distribution of these two newly identified genotypes.

We found that more than half (65%) of the norovirus-positive packages contained two or more norovirus genotypes (Table 1 and Fig. 1). Multiple norovirus genotypes have also been found in oyster-associated outbreaks of gastroenteritis *(10)*, and in a recent study, we found multiple norovirus genotypes in outbreaks of gastroenteritis at various food-catering settings throughout Japan *(22)*. These findings indicate that like oyster-associated outbreaks, clam-associated outbreaks may also be caused by multiple norovirus genotypes, although further studies are needed.

The Aichi virus was found in 33% of the clam packages, and all of these packages were co-contaminated with other viruses. The Aichi virus sequences detected in the packages closely matched other Aichi virus sequences (genogroup A) that were detected in patient stool specimens from oyster-associated gastroenteritis *(26)*. To the best of our knowledge, these results have shown for the first time that the Aichi virus can also accumulated in these Japanese clams. The importance of Aichi virus in human gastroenteritis is still poorly understood, and very few studies have reported Aichi virus infections since its first discovery in 1989 *(25)*. One recent study detected Aichi virus in only 3% (28 of 912) of stool specimens from infants with sporadic cases of gastroenteritis (collected in Japan, Bangladesh, Thailand, and Vietnam), which were negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus *(23)*. Further studies are clearly needed in order to determine the importance of this virus in humans.

Rotavirus was detected in 14 of 33 available clam packages. A similar study in France found rotavirus in 52% of mussel samples and 27% of oyster samples *(15)*. Rotavirus infections usually causes sporadic cases of gastroenteritis in children in the winter season, but our results suggest that rotavirus may persist longer in the environment, at least up to June (Table 1). A great genetic diversity of rotavirus G types was detected in the packages, and we also found that 9 of 17 rotavirus-positive packages contained two or more rotavirus G types. Likewise, a study in Egypt and Spain also found a great genetic diversity of rotavirus G types as well as unusual genotypes in sewage samples *(24)*.

Enteric adenoviruses (Ad40 and Ad41) were detected in 17 of 33 available clam packages. Adenovirus infections in the western part of Japan were reported to be low, with one study reporting adenovirus serotype 41 in only approximately 3% of stool specimens from infants with sporadic cases of gastroenteritis *(6)*. The high detection rate of adenoviruses in these packages may indicate that adenovirus prevalence is variable, although further studies are needed.

One (2%) of 57 packages was contaminated with HAV, and sequence analysis indicated that it belonged to subtype IA. The low detection rate of HAV was also observed in an oyster study that found only 2 of 112 samples positive in Japan *(12)*. The low detection rate of HAV in the clams and oysters was not unusual, because the prevalence of HAV infections is low in Japan, although this may be increasing *(13)*. More surveillance is clearly needed in order to locate other contaminated areas and help control the spread of HAV contamination.

Astroviruses were not detected in any of the Japanese clam packages. This result is surprising because astroviruses were detected in more than half (61%) of African clam samples *(5)*, 50% of French mussel samples *(15)*, and 17% of French oyster samples *(15)*. This result suggested that the astrovirus may not concentrate to detectable levels in certain species of shellfish or the level of contamination differs in each place, which was similarly observed in two other studies *(3, 21)*.

In conclusion, this study has shown that the Japanese clams were highly contaminated with many types of human enteric viruses capable of causing gastroenteritis and/or acute viral hepatitis. At present, the Enforcement Regulation of Food Sanitation Law mainly focuses on bacterial contamination in Japan *(21)*. Clearly, regulations and standards need to be revised in order to address this problem of viral contamination in the Japanese clams. The health risks associated with eating contaminated oysters have been well documented, but further studies are clearly needed in order to determine the health risks associated with eating these contaminated Japanese clams.

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Acute Gastroenteritis Caused by GI/2 Sapovirus, Taiwan, 2007

To the Editor: Sapovirus is an etiologic agent of human gastroenteritis. Although many of the previously reported cases were of mild, sporadic infections in young children (*1–3*), several recent sapovirus-associated gastroenteritis outbreaks have affected adults, which suggests that the virus's virulence, prevalence, or both, may be increasing (*4–6*). In this study, we describe a sapovirus-associated outbreak of gastroenteritis that occurred during May 4–8, 2007, and involved college students in northern Taiwan.

A total of 55 students had clinical symptoms of gastroenteritis, including diarrhea (45), vomiting (22), abdominal cramps (17), and fever (2). The clinical symptoms continued for up to 10 days (mean 4.7 days). Stool

specimens were collected from 8 of 55 students on May 8 (Table). Initially, the specimens were screened for bacteria, rotavirus, and norovirus, but all specimens were negative for these pathogens. The 8 stool specimens were then examined by electron microscopy (EM), and 1 was positive for calicivirus-like particles.

To confirm the EM results, we performed reverse transcription–PCR (RT-PCR), real-time RT-PCR, and sequence analysis as previously described (*7*). Briefly, purified RNA (10 μL) was reverse transcribed by using SuperScript III reverse transcriptase according to the manufacture's instructions (Invitrogen, Carlsbad, CA, USA). PCR was carried out by using the SV-F11 and SV-R1 primer set directed against the conserved N terminal capsid region (*8*). The PCR products were analyzed with 2% agarose gel electrophoresis and visualized after ethidium bromide staining. The PCR-generated amplicons (≈780 bp) 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) and determined with the ABI 3130 sequencer (Applied Biosystems, Foster City, CA, USA.). Nucleotide sequences were aligned by using ClustalX (www.clustal.org), and the distances were calculated by using the Kimura 2-parameter method. A phylogenetic tree was generated by the neighbor-joining method as described previously (*1*,*8*).

Of the 8 specimens, 7 were positive by RT-PCR and real-time RT-PCR (Table). SaV124F, SaV1F, SaV5F, and SaV1245R primers as well as SaV124TP and SaV5TP minor-groove binding probes were used for real-time RT-PCR diagnosis, which targets the sapovirus RdRp-capsid junction region as described (*7*). The number of sapovirus cDNA copies ranged from 2.86×10^8 to 1.72×10^{10} copies/g of stool specimen; mean was 2.71×10^9 copies/g of stool specimen (Table). Sequence analysis of the 7 positive specimens showed 100% nucleotide identity (nt 5098–5878), indicating that the outbreak was caused by 1 sapovirus strain.

To better classify the sapovirus, we reamplified the 3′ end of the genome from 1 positive specimen and sequenced ≈2,400 nt (nt 5074-3′) (Hu/SaV/9–5/Taipei/07/TW; GenBank accession no. EU124657). PCR was performed with SV-F13, SV-F14, and TX30SXN primers as described (*1*). Database searches found a closely matching sapovirus sequence (99%) that was detected in a patient with gastroenteritis in Japan, in 2004 (Chiba041413 strain; GenBank accession no. AB258427). The next closely matching sequence was detected in an outbreak of gastroenteritis among adults in the United States in 1994 (Parkville strain; HCU73124) (*6*). Phylogenetic analysis clustered these 3 sapovirus sequences into genogroup I/genotype 2 (GI/2) (online Appendix Figure, available from www.cdc.gov/content/EID/14/7/1169-appG.htm).

Sapovirus was reported in Japan in water samples (untreated wastewater, treated wastewater, and a river) and in clam samples intended for human consumption (*1*). Apart from these 2 environmental studies, little is known about reservoir of sapovirus or its route of infection in the natural environment. The source of contamination in this current outbreak was not determined; however, none of the food handlers associated with the college reported symptoms of gastroenteritis. However, in a recent molecular epidemiologic study in Japan, a large number of symptomatic and asymptomatic food handlers were found to be infected with noroviruses (*9*). Several seroprevalence studies also indicated high prevalence rates of antibodies to sapovirus in adults and children (*10*). All of these findings highlight the need to collect stool specimens from asymptomatic persons and indicate possible "silent" transmission through an asymptomatic route. Symptoms of sapovirus infection are thought to be milder than symptoms of norovirus infections. However, in this study approximately one third (17) of the 55 students reported symptoms of abdominal pain and 22 (40%) reported symptoms of vomiting. Many of the earlier sapovirus studies described sapovirus GI/1 infections in young Japanese children (*1*), which indicated that infecting virus had a different genotype than the virus detected in this study (GI/2).

Table. Clinical symptoms and laboratory diagnosis results for sapovirus-related outbreak among college students, northern Taiwan, May 2007*†

Specimen no.	Patient sex/age, y	Date of illness onset	EM results	RT-PCR results	Copies cDNA/g of stool‡	Symptom			
						Fever	Diarrhea	Vomiting	Abdominal pain
1	F/20	May 5	–	+	1.69×10^8	–	+	+	+
2	F/26	May 5	–	+	6.19×10^8	–	+	+	–
3	M/19	May 6	–	+	2.32×10^8	–	+	–	+
4	M/18	May 6	–	+	3.24×10^8	–	+	+	+
5	F/21	May 7	+	+	1.72×10^{10}	–	+	–	–
6	F/18	May 4	–	–	–	+	+	+	–
7	M/19	May 7	–	+	4.28×10^8	–	+	+	+
8	F/20	May 6	–	+	2.86×10^7	–	+	–	+

*EM, electron microscopy; RT-PCR, reverse transcription–PCR; –, negative; +, positive.
†All specimens were collected May 8.
‡cDNA copies were determined by real-time PCR.

In addition, the viral load in this study appeared to be comparatively high. These results suggest that some sapovirus genotypes are more virulent than others. Similar findings were obtained with norovirus infections around the world; strains belonging to norovirus GII/4 were the most prevalent in many countries. Although several recombinant sapovirus strains have been identified and found to be the cause of increased numbers of infections in some countries (*1*,*5*), they were not observed in this study. Increased sapovirus surveillance and reporting are needed to shed some more light on this poorly understood virus.

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NOTE

Development of a broadly reactive nested reverse transcription-PCR assay to detect murine noroviruses, and investigation of the prevalence of murine noroviruses in laboratory mice in Japan

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ABSTRACT

A broadly reactive nested RT-PCR assay to detect MNV was developed and subsequently used to investigate the prevalence of MNV in laboratory mice in Japan. MNV were detected in 8 (22%) of 37 murine stool specimens by second-round PCR, although no positive band was obtained from any specimen by first-round PCR. Genetic analysis of the second round PCR products showed that MNV sequences detected in this study were closely matched (97.2~99.1%) to that of MNV-3 (DQ223042). This is the first report demonstrating the prevalence of MNV in Japan.

Key words laboratory mice, murine noroviruses, nested RT-PCR, phylogenetic analysis.

Noroviruses are single-stranded, positive-sense RNA viruses that belong to the genus *Norovirus* in the family *Caliciviridae* (1). These viruses are currently divided into five genetically distinct genogroups: GI to GV. Most human noroviruses which cause outbreaks of gastroenteritis are classified as GI and GII, whereas animal noroviruses which infect swine and cattle are classified as GII and GIII, respectively (2–4). Recently, a murine norovirus (MNV) which infects laboratory mice was discovered and designated as a member of a new genogroup (GV) within the genus *Norovirus* (5). Because MNV is the only norovirus that replicates efficiently *in vitro* (6), it has been used to study proteolytic processing, survival in environments, and reverse genetics systems (7–9).

Many MNV infections, including those naturally occurring in laboratory mice, have recently been reported (10), and MNV is recognized as one of the most prevalent pathogens in research mice. As new strains of MNV in laboratory mice have been isolated and characterized, it has been discovered that a large number of genetically diverse MNV strains exist (11, 12). For most biomedical research, which is highly dependent on mice as experimental models, MNV infection among laboratory mice is of great concern. Therefore, sensitive diagnostic

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List of Abbreviations: GI, genogroup I; GII, genogroup II; GIII, genogroup III; GIV, genogroup IV; GV, genogroup V; MNV, murine norovirus(es); ORF, open reading frame; PBS, phosphate-buffered saline; RdRp, RNA-dependent RNA polymerase; RT-PCR, reverse transcription-polymerase chain reaction; UV, ultraviolet.

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Table 1 Primers used to detect murine noroviruses by nested RT-PCR

	Primer	Sequence (5′–3′)	Polarity[a]	Location[b]
1st PCR (Outer)	MNV-F1	GCCATGCATGGTGAAAAG	+	4887–4904
	MNV-R1	CATGCARACCAGGCGCATAG	−	5588–5607
2nd PCR (Inner)	MNV-F2	ACARTGGATGCTGAGACC	+	5010–5027
	MNV-R2	CAACCACCTTGCCAGCAG	−	5408–5425

[a]+, sense; −, anti-sense.
[b]Corresponding nucleotide position of MNV-1 strain complete genome (AY228235).

assays to detect MNV in laboratory mice are greatly needed.

In this study, we developed a broadly reactive nested RT-PCR assay to detect all currently known MNV strains, and subsequently used the assay to investigate the prevalence of MNV in laboratory mice in Japan.

Full- or near full-length genome nucleotide sequences of 42 MNV strains were aligned using GENETYX Mac (Genetyx Corporation, Tokyo, Japan). The primer sets were designed to be able to detect a broad range of currently known MNV strains. The accession numbers of the MNV sequences used were as follows: EF014462, EU004659, NC008311, EU482057, DQ285629, EU004654, EU004672, EU482058, EU004681, EU004658, EU004675, EU004656, EU004678, EU004668, EU004664, EU004679, EU004680, EU004655, EU004671, EU004660, EU004662, DQ223043, EF531291, EU004676, EU004667, EU004666, EU004665, EU004682, DQ223041, EU004669, EU004657, AY228235, EU004661, EU004673, EU004674, EU004677, EU004670, EF531290, EU004683, DQ911368, EU004663, and DQ223042.

Thirty-seven murine stool specimens were collected from six laboratories in Japan. Approximately 0.1g of the murine stool specimens was suspended in 0.9ml of PBS and centrifuged at 15000rpm for 5 min. Viral RNA was extracted from the supernatant using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. To prevent non-specific amplification, the extracted viral RNA was treated with DNase I before RT reaction. Viral RNA (10μl) was added to a reaction mixture (5μl) containing DNase I buffer (150 mM Tris-HCl, pH 8.3; 225 mM KCl, 9 mM $MgCl_2$) and 1U of RQ1 DNase (Promega, Madison, WI, USA). The reaction mixture was incubated at 37°C for 30 min to digest DNA and then at 75°C for 5 min to inactivate the enzyme. The RT reaction was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Briefly, DNase I–treated RNA (15 μl) was added to 15μl of RT mixture, which contained 75U of MultiScribe Reverse Transcriptase and random hexamers (Applied Biosystems). The RT reaction mixture was incubated at 25°C for 10 min, then at 37°C for 120 min, and then at 85°C for 5 min to inactivate the enzyme.

The first PCR was carried out in 50 μl of reaction volume containing 5 μl of cDNA, 2.5 U of Ex *Taq* DNA polymerase (Takara Bio, Shiga, Japan), and 20pmol of outer primer set (Table 1). PCR amplification was performed under the following conditions: initial denaturation at 94°C for 3 min to activate DNA polymerase, followed by 40cycles of amplification with denaturation at 94°C for 30sec, primer annealing at 50°C for 30sec, extension reaction at 72°C for 1 min, and then a final extension at 72°C for 7 min. The second PCR was performed with 2 μl of the first PCR product and 48 μl of the reaction mixture containing the inner primer set (Table 1), under conditions identical to those of the first PCR. The PCR products were separated by electrophoresis in a 2% agarose gel and visualized under a UV lamp after ethidium bromide staining.

The nested PCR products of the MNV-positive samples were purified using a QIAquick PCR purification kit (Qiagen) and then both strands of the purified PCR product were sequenced directly with a BigDye Cycle Sequencing Kit and 3130 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were aligned with Clustal W, and distances were calculated with Kimura's 2-parameter method (13). A phylogenetic tree from bootstrap analysis with 1000 replicates was generated by the Neighbor-Joining method. The nucleotide sequences determined in this study have been submitted to GenBank under accession numbers AB490075–AB490082.

On the basis of the nucleotide sequences of the 42 MNV strains, we designed nested PCR primer sets within conserved sequences of the MNV genome to react with all currently known MNV strains. As shown in Figure1, the outer primer set was designed to generate a 721-bp product corresponding to the nucleotide position of 4887–5607, which is located on the 3′ terminus of ORF1 and the 5′ terminus of ORF2, whereas the inner primer set was designed to generate a 416-bp product of the capsid region corresponding to the nucleotide position of 5010–5425. The cross-reactivity of these primer sets was evaluated with stool specimens containing other caliciviruses, such

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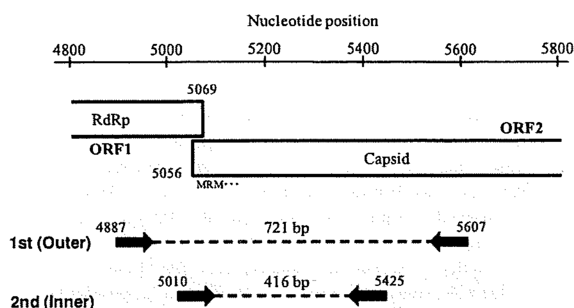

Fig. 1. Location of the primers in the ORF1-ORF2 junction region of the MNV genome. The primers are identified by black arrows.

as noroviruses (GI, GII) and sapoviruses (GI, GII, GIV, GV). Non-specific amplification was not observed (data not shown). Furthermore, a nucleotide BLAST search of each primer showed no significant homology to non-MNV sequences. Therefore, the primer sets used in this study were considered to be specific to MNV genomes.

Thirty-seven murine stool specimens collected from six separate laboratory mouse colonies in Japan, were screened for MNV using the newly developed nested RT-PCR assay. MNV was detected in 8 (22%) of 37 murine stool specimens by second-round PCR. Genetic analysis of the second round PCR products showed that all of the MNV sequences detected in this study were closely matched (97.2~99.1%) to that of MNV-3 (DQ223042), which was discovered by Hsu *et al.* (4) (Fig. 2), and had completely identical amino acid sequences (data not shown).

Until now, MNV strains have been isolated from laboratory mice in only a few countries, such as the USA and Germany (5, 11, 12, 14–17). This is the first report describing the prevalence of MNV in Japan. Although the prevalence of MNV in laboratory mice is not well known, the results of the present study suggest the possibility of widespread MNV infection in laboratory mice in Japan.

When the single-round one-step RT-PCR developed by Hsu *et al.* (4) was examined with our specimens, positive bands were obtained from only 2 (5.4%) of 37 specimens (data not shown). In addition, the nested RT-PCR showed a much higher positive rate than single-round RT-PCR using outer or inner primer pairs designed in this study (data not shown).

Evidence of recombination among MNV genomes suggests that MNV are continually evolving; therefore, additional genetically divergent MNV strains likely exist (11, 12). However, the 5′ terminus of the capsid gene sequence is highly conserved among all known MNV strains, as previously described (12). Herein we describe a nested RT-PCR assay targeting this region to detect a broad range of currently known MNV genomes. In this study, we designed new primer sets to detect all currently known murine norovirus strains deposited on the nucleotide database, and succeeded in identifying several unique Japanese strains using this nested RT-PCR.

MNV is a newly recognized infectious agent in laboratory mice and methods for eliminating MNV from

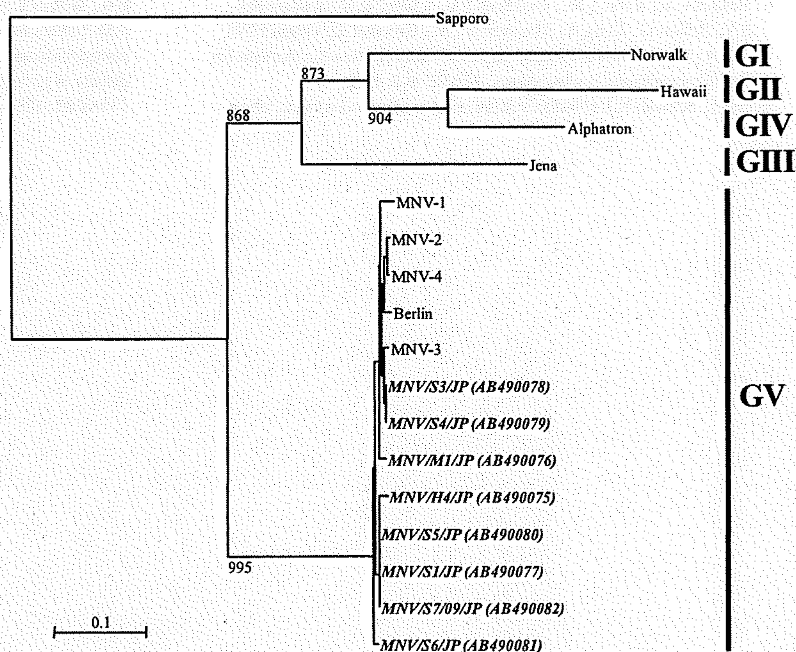

Fig. 2. Phylogenetic dendrogram based on the 5′ terminus of the capsid gene of noroviruses (approximately 350 nucleotides) showing several genetically distinct genogroups. Items in ***Italic Bold*** are MNV sequences detected in this study and GenBank accession numbers. The numbers on each branch indicate the bootstrap values. Bootstrap values of 950 or higher were considered statistically significant for the grouping. The scale bar represents nucleotide substitutions per site. The Sapporo virus of the genus *Sapovirus* was used as an outgroup. GenBank accession numbers for the reference strains are as follows: Sapporo, U65427; Norwalk, M87661; Hawaii, U07611; Jena, AJ011099; Alphatron, AF195847; MNV-1, AY228235; MNV-2, DQ223041; MNV-3, DQ223042; MNV-4, DQ223043; Berlin, DQ911368.

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research mouse colonies have not been established. The development of a sensitive and specific diagnostic assay is greatly needed to identify MNV-infected mice. Our broadly reactive nested RT-PCR assay will be a powerful tool for estimating the prevalence of MNV infections in laboratory mice and for establishing MNV-free mouse colonies.

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