

## Research Note

# Detection of Human Enteric Viruses in Japanese Clams

GRANT S. HANSMAN,<sup>1\*</sup> TOMOICHIRO OKA,<sup>1</sup> TIAN-CHENG LI,<sup>1</sup> OSAMU NISHIO,<sup>2</sup> MAMORU NODA,<sup>3</sup> AND  
 NAOKAZU TAKEDA<sup>1</sup>

<sup>1</sup>Department of Virology II and <sup>2</sup>Infectious Diseases Surveillance Center, National Institute of Infectious Diseases, Musashi-murayama, Tokyo 208-0011, Japan; and <sup>3</sup>National Institute of Health Sciences, Kami-Yoga 1-18-1, Setagaya-ku, Tokyo, Japan

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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.

## MATERIALS 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  $10,000 \times g$  for 30 min at 4°C, the supernatant was layered onto 1 ml of 30% sucrose solution and ultracentrifuged at  $154,000 \times g$  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 QIAamp 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

\* Author for correspondence. Tel: +81-42-561-0771; Fax: +81-42-561-4729; E-mail: ghanzman@nih.go.jp.

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	GI-Shijimi3 (GI/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	GI-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	GI-Shijimi14 (GII/New)	—	G4	+	—	—	—
18	C	02/25/06	1	GI-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

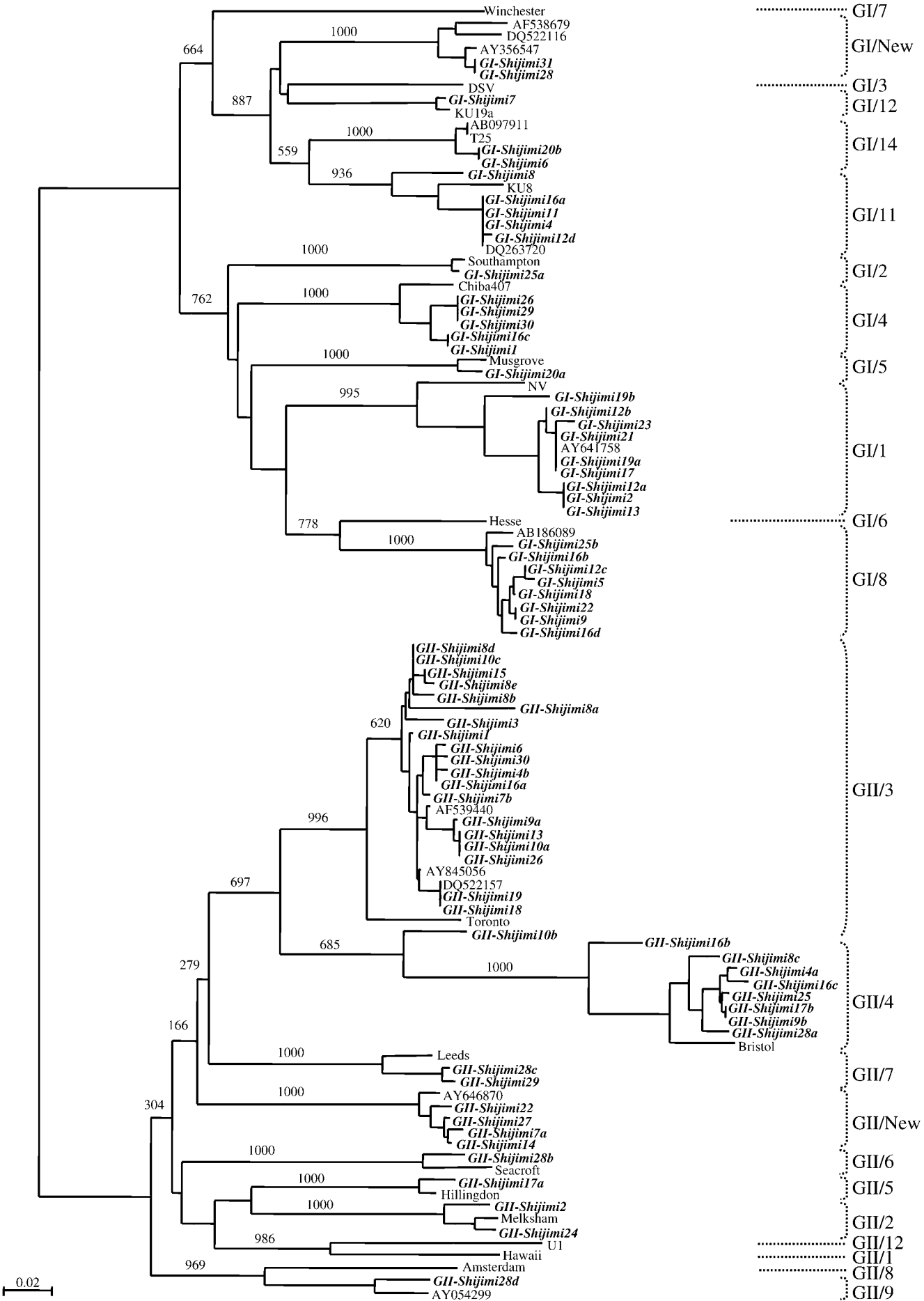
Package no.	Site	Mo/day/yr	No. of norovirus genotypes	Norovirus strain name (genogroup/genotype)	Aichi virus	Rotavirus: G types	Adeno-virus	HAV	HEV (from (17))	Sapovirus (from (9))
31	G	03/18/06	1	GI-Shijimi23 (GI/1)	—	NT	NT	—	—	—
32	H	03/18/06	—		—	NT	NT	—	—	—
33	D	03/30/06	1	GII-Shijimi24 (GII/2)	Aic-15	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	—	— <sup>b</sup>	—
48	D	07/21/06	—		—	NT	NT	—	— <sup>b</sup>	—
49	B	07/21/06	—		—	NT	NT	—	— <sup>b</sup>	—
50	A	07/27/06	—		—	NT	NT	—	— <sup>b</sup>	—
51	A	08/06/06	—		—	NT	NT	—	— <sup>b</sup>	—
52	D	08/07/06	—		—	NT	NT	—	— <sup>b</sup>	—
53	B	08/10/06	—		—	NT	NT	—	— <sup>b</sup>	—
54	D	08/23/06	—		—	NT	NT	—	— <sup>b</sup>	—
55	I	09/04/06	—		—	NT	NT	—	— <sup>b</sup>	—
56	B	09/04/06	1	GI-Shijimi31 (GI/New)	—	NT	NT	—	— <sup>b</sup>	—
57	D	09/06/06	—		—	NT	NT	—	— <sup>b</sup>	—
Total				31/57	19/57	17/33	17/33	1/57	2/46	4/57

<sup>a</sup> NT, not tested.  
<sup>b</sup> 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 cap-

sid 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 amplify 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-



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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