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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	−	−	−

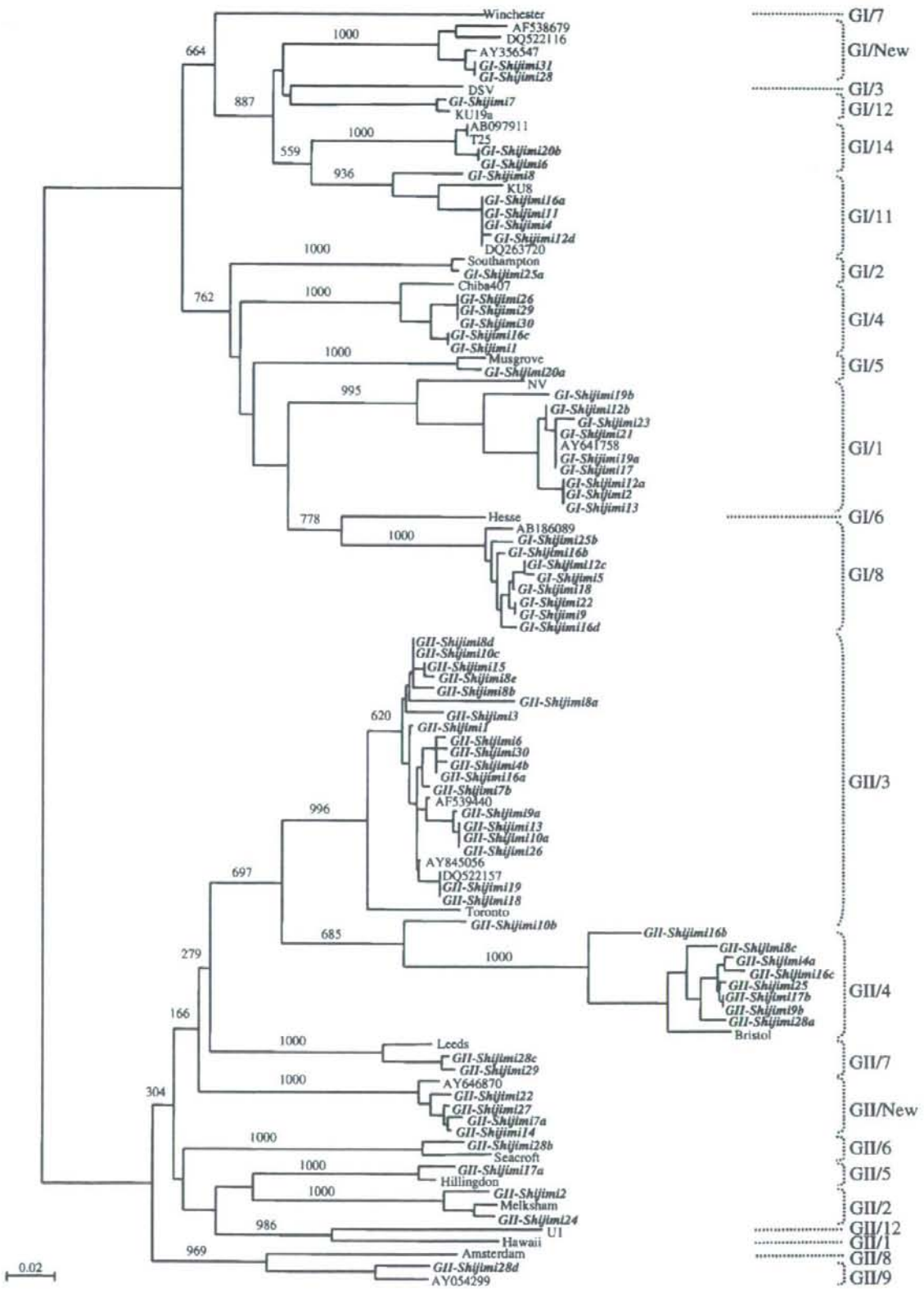
TABLE 1. *Continued*

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
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

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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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REFERENCES

1. Allard, A., B. Albinsson, and G. Wadell. 1992. Detection of adenoviruses in stools from healthy persons and patients with diarrhea by two-step polymerase chain reaction. *J. Med. Virol.* 37:149–157.
2. Bull, R. A., E. T. Tu, C. J. McIver, W. D. Rawlinson, and P. A. White. 2006. Emergence of a new norovirus genotype II.4 variant associated with global outbreaks of gastroenteritis. *J. Clin. Microbiol.* 44:327–333.
3. Costantini, V., F. Loisy, L. Joens, F. S. Le Guyader, and L. J. Saif. 2006. Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. *Appl. Environ. Microbiol.* 72:1800–1809.
4. Di Pinto, A., M. C. Conversano, V. T. Forte, G. La Salandra, C. Montervino, and G. M. Tantillo. 2004. A comparison of RT-PCR-based assays for the detection of HAV from shellfish. *New Microbiol.* 27:119–124.
5. Elamri, D. E., M. Aouni, S. Parnaudeau, and F. S. Le Guyader. 2006.

Detection of human enteric viruses in shellfish collected in Tunisia. *Lett. Appl. Microbiol.* 43:399–404.
6. Fukuda, S., M. Kuwayama, S. Takao, Y. Shimazu, and K. Miyazaki. 2006. Molecular epidemiology of subgenus F adenoviruses associated with pediatric gastroenteritis during eight years in Hiroshima Prefecture as a limited area. *Arch. Virol.* 151:2511–2517.
7. Gouvea, V., R. I. Glass, P. Woods, K. Taniguchi, H. F. Clark, B. Forrester, and Z. Y. Fang. 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J. Clin. Microbiol.* 28:276–282.
8. Hansman, G. S., L. T. Doan, T. A. Kguyen, S. Okitsu, K. Katayama, S. Ogawa, K. Natori, N. Takeda, Y. Kato, O. Nishio, M. Noda, and H. Ushijima. 2004. Detection of norovirus and sapovirus infection among children with gastroenteritis in Ho Chi Minh City, Vietnam. *Arch. Virol.* 149:1673–1688.
9. Hansman, G. S., T. Oka, R. Okamoto, T. Nishida, S. Toda, M. Noda, D. Sano, Y. Ueki, T. Imai, T. Omura, O. Nishio, H. Kimura, and N. Takeda. 2007. Human sapovirus in clams, Japan. *Emerg. Infect. Dis.* 13:620–622.
10. Kageyama, T., M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, S. Kojima, R. Takai, T. Oka, N. Takeda, and K. Katayama. 2004. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to Norovirus in Japan. *J. Clin. Microbiol.* 42:2988–2995.
11. Katayama, K., H. Shirato-Horikoshi, S. Kojima, T. Kageyama, T. Oka, F. Hoshino, S. Fukushi, M. Shinohara, K. Uchida, Y. Suzuki, T. Gojobori, and N. Takeda. 2002. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. *Virology* 299:225–239.
12. Kitahashi, T., T. Tanaka, and E. Utagawa. 1999. Detection of HAV, SRSV and astrovirus genomes from native oysters in Chiba City, Japan. *Kansenshogaku Zasshi* 73:559–564.
13. Kiyohara, T., T. Sato, A. Totsuka, T. Miyamura, T. Ito, and T. Yoneyama. 2007. Shifting seroepidemiology of hepatitis A in Japan, 1973–2003. *Microbiol. Immunol.* 51:185–191.
14. Kojima, S., T. Kageyama, S. Fukushi, F. B. Hoshino, M. Shinohara, K. Uchida, K. Natori, N. Takeda, and K. Katayama. 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J. Virol. Methods* 100:107–114.
15. Le Guyader, F., L. Haugarreau, L. Miossec, E. Dubois, and M. Pommepuy. 2000. Three-year study to assess human enteric viruses in shellfish. *Appl. Environ. Microbiol.* 66:3241–3248.
16. Le Guyader, F., F. H. Neill, M. K. Estes, S. S. Monroe, T. Ando, and R. L. Atmar. 1996. Detection and analysis of a small round-structured virus strain in oysters implicated in an outbreak of acute gastroenteritis. *Appl. Environ. Microbiol.* 62:4268–4272.
17. Li, T. C., T. Miyamura, and N. Takeda. 2007. Detection of hepatitis E virus RNA from the bivalve Yamato-Shijimi (*Corbicula japonica*) in Japan. *Am. J. Trop. Med. Hyg.* 76:170–172.
18. Matsui, M., H. Ushijima, M. Hachiya, J. Kakizawa, L. Wen, M. Oseto, K. Morooka, and J. Kurtz. 1998. Determination of serotypes of astroviruses by reverse transcription-polymerase chain reaction and homologies of the types by the sequencing of Japanese isolates. *Microbiol. Immunol.* 42:539–547.
19. Murray, C. J., and A. D. Lopez. 1996. Evidence-based health policy—lessons from the Global Burden of Disease Study. *Science* 274: 740–743.
20. Nishida, T., H. Kimura, M. Saitoh, M. Shinohara, M. Kato, S. Fukuda, T. Munemura, T. Mikami, A. Kawamoto, M. Akiyama, Y. Kato, K. Nishi, K. Kozawa, and O. Nishio. 2003. Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters. *Appl. Environ. Microbiol.* 69:5782–5786.
21. Nishida, T., O. Nishio, M. Kato, T. Chuma, H. Kato, H. Iwata, and H. Kimura. 2007. Genotyping and quantitation of noroviruses in oysters from two distinct sea areas in Japan. *Microbiol. Immunol.* 51: 177–184.
22. Ozawa, K., T. Oka, N. Takeda, and G. S. Hansman. 2007. Norovirus infections in symptomatic and asymptomatic food-handlers in Japan. *J. Clin. Microbiol.* 45:3996–4005.
23. Pham, N. T., P. Khamrin, T. A. Nguyen, D. S. Kanti, T. G. Phan, S. Okitsu, and H. Ushijima. 2007. Isolation and molecular characterization of Aichi viruses from fecal specimens collected in Japan, Bangladesh, Thailand, and Vietnam. *J. Clin. Microbiol.* 45:2287–2288.
24. Villena, C., W. M. El-Senousy, F. X. Abad, R. M. Pinto, and A. Bosch. 2003. Group A rotavirus in sewage samples from Barcelona and Cairo: emergence of unusual genotypes. *Appl. Environ. Microbiol.* 69:3919–3923.
25. Yamashita, T., S. Kobayashi, K. Sakae, S. Nakata, S. Chiba, Y. Ishihara, and S. Isomura. 1991. Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. *J. Infect. Dis.* 164:954–957.
26. Yamashita, T., M. Sugiyama, H. Tsuzuki, K. Sakae, Y. Suzuki, and Y. Miyazaki. 2000. Application of a reverse transcription-PCR for identification and differentiation of Aichi virus, a new member of the picornavirus family associated with gastroenteritis in humans. *J. Clin. Microbiol.* 38:2955–2961.

NOTE *Virology*

Mice are Not Susceptible to Hepatitis E Virus Infection

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ABSTRACT. To determine whether or not mice are susceptible to hepatitis E virus (HEV) infection, C57BL/6 mice were experimentally infected with genotypes 1, 3 and 4 HEV by intravenous injection. Serum and stool samples were collected and used to detect HEV RNA and anti-HEV antibodies by RT-PCR and ELISA. The virus infection was monitored up to two months after inoculation; however, none of the serum or stool samples was positive for virus replication, demonstrating that C57BL/6 mice were not susceptible to HEV.

KEY WORDS: C57BL/6, hepatitis E, hepatitis E virus, HEV, mouse.

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Hepatitis E is a serious public health concern in many developing countries, and recognized as sporadic and endemic acute hepatitis in many industrialized countries. Pregnant women have a high risk associated with hepatitis E, with a high mortality rate (up to 20%) [5, 25]. The causative agent of hepatitis E is hepatitis E virus (HEV), and this virus transmits primarily via the fecal-oral route through contaminated drinking water [1, 6]. HEV is the sole member of the genus *Hepevirus* in the family *Hepeviridae*. HEV is a small round non-enveloped virus, 27–34 nm in diameter, containing an RNA genome approximately 7.2 kb in length [2, 3]. The RNA consists of a single-strand RNA molecule containing three discontinuous and partially overlapping open reading frames (ORFs). The 3' terminus of the RNA is polyadenylated. HEV isolates were grouped into at least four major genotypes, genotypes 1, 2, 3 and 4 (G1, G2, G3 and G4) on the basis of nucleotide and deduced amino acid sequences [3, 6, 24]. Because G3 and G4 HEV were isolated from pigs and wild boars in addition to humans, and much direct and indirect evidence has indicated that HEV transmits from pigs or wild boars to humans, hepatitis E is recognized as a zoonotic disease [8, 18, 23]. Many studies have reported the detection of HEV RNA and the HEV-specific antigen (HEV-Ag) in pig and wild boar stool and serum specimens, and suggested the active circulation of this virus among these animals [18, 20, 26]. HEV-specific antibodies have been detected in many animals including sheep, cows, dogs, cats, wild rats, wild deer and mongoose, in addition to pigs and wild boars [9, 12, 14, 15, 19]. However, it is obscure whether or not HEV substantially replicates in these animals. In this study we infected C57BL/6 mice with G1, G3 and G4 HEV, and monitored the virus growth to determine the susceptibility of mice to HEV infection.

G1 HEV strain was derived from stool specimens from a cynomolgus monkey (*Macaca fascicularis*), born and grown in the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases (NIID), which had been experimentally infected with an Indian strain [10]. The G3 HEV strain (DQ079632) was derived from stool specimens collected on a pig farm in Japan. The G4 HEV strain (DQ079628) was from a stool specimen collected from a wild boar caught in Aichi prefecture, Japan. The stool specimens were used to prepare 10% (w/v) suspensions as described [10]. These suspensions were positive for HEV RNA by reverse-transcription polymerase chain reaction (RT-PCR). The concentrations of the G1, G3, and G4 HEV were 5×10^4, 2×10^4 and 1×10^5 copies per one m*l* of suspension, respectively, by real time RT-PCR (unpublished).

To confirm the infectivity of these stool specimens, 3 cynomolgus monkeys (4 year-old males) were inoculated intravenously with 2 m*l* of one of the suspensions, and the stools were collected daily, and used to detect HEV RNA and HEV-Ag. Sera were collected weekly before and after the inoculation to detect HEV RNA, HEV-Ag, and HEV-specific IgG antibodies. The sera were also used to determine ALT values. All monkey experiments were reviewed by the Institute's ethical committee and carried out according to "Guides for animal experiments performed at NIID" under codes 990058, 000019 and 504006. The primates were individually housed in BSL-2 facilities. Detection of HEV RNA, HEV-Ag, and IgG has been described previously [8, 10, 11]. The ALT value was measured as described [10]. As shown in the figure, HEV RNA and HEV-Ag were detected within one week in the sera (A) and stools (B) of all three monkeys after inoculation, and ALT values increased more than three-fold compared with that of pre-inoculation in infected monkeys, though the increase was slow and the values were low in G1 HEV- and G3 HEV-infected animals (C), indicating that all three HEV strains, G1, G3 and G4, were infectious. Furthermore, drastic increases of IgG antibody titers, probably due to extensive replication of the virus, were demonstrated in these animals (D). These results confirmed that the HEVs used in these experiments were indeed infectious.

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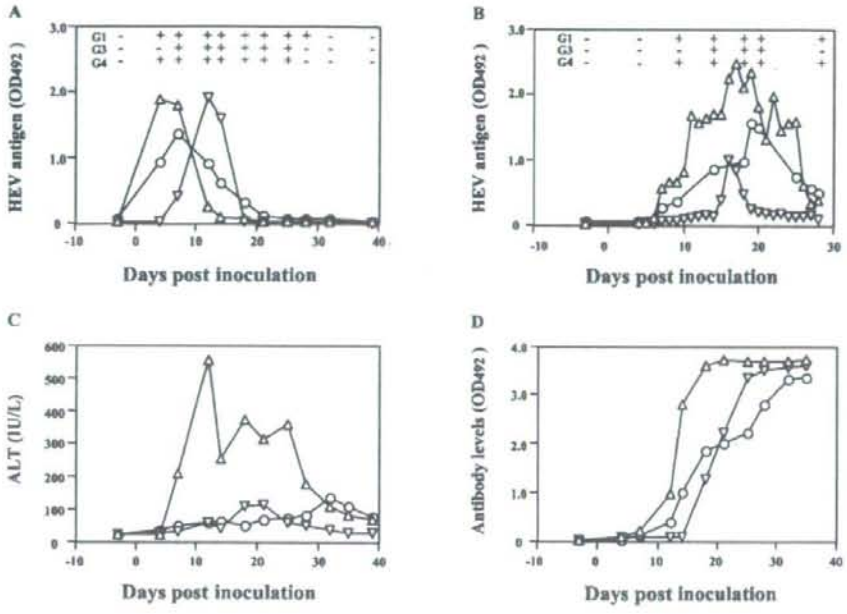

Fig. 1. Kinetics of biochemical, serological, and virological markers in monkeys after inoculation. HEV-Ag and HEV RNA in sera (A) and stools (B), was measured by an antigen ELISA and RT-PCR. ALT was indicated (C), and IgG antibody was measured by an antibody-ELISA (D). ○, monkey inoculated with G1 HEV; ▽, monkey inoculated with G3 HEV; △, monkey inoculated with G4. HEV RNA was monitored by RT-PCR . + , Positive; − , Negative.

Six 4-week-old and five 9-month-old C57BL/6 mice, negative for anti-HEV antibody and HEV RNA, were inoculated intravenously with 100 μl of 10% fecal suspensions. Serum and fecal samples were obtained at 1 week before, and at 1, 2, 3, 4, 6, 8, and 10 weeks after inoculation, and HEV RNA and anti-HEV IgG antibodies were measured. However, neither serum nor fecal specimens were positive for HEV infection, clearly indicating that HEV did not replicate in C57BL/6 mice (data not shown). In other words, the C57BL/6 mice were not susceptible to hepatitis E virus.

Anti-HEV IgM and/or IgG antibody and HEV RNA are frequently detected in pigs and wild boars in various countries, and these 2 animals are recognized as the main reservoirs of HEV. Although the infection is asymptomatic when G3 and G4 HEVs are used to inoculate pigs, it is obvious that pigs are susceptible to HEV infection [17]. Interestingly, pigs were resistant to experimental infection with G1 and G2 HEVs [16]. Although experimental data is not available for wild boars, these animals are genetically close to pigs, and wild boars are likely to be susceptible to HEV. Direct and indirect evidence of HEV transmission from wild boars and pigs to humans has been reported in Japan, suggesting that these animals are the main zoonotic reservoirs in this country [8, 27]. Chimpanzees, rhesus monkeys, cynomolgus monkeys, and marmosets have been used for experimental infection and to evaluate the efficacy of HEV vaccines, and HEV has been used as a challenge virus, indicating that these monkeys are susceptible to HEV infection [13, 22, 29, 30]. In addition to these animals, anti-HEV IgG antibody has been detected in dogs, cats, cows, goats, sheep, and rodents including rats [4, 7, 14, 19], and anti-HEV IgG antibody and HEV RNA were detected from mongoose and wild deer [9, 21, 28]. However, the susceptibility of these animals to HEV infection has not been fully evaluated, and whether or not HEV replicates *in vivo* in these animals is unknown.

We evaluated the susceptibility of B57C/6 mice by directly inoculating infectious HEV through intravenous injection. Although two different age groups, at 4 weeks and 9 months, were used, none of the mice was successful in producing *in vivo* HEV replication. Our study clearly demonstrated that C57BL/6 mice are resistant to HEV infection. By contrast, our preliminary results indicated that HEV is capable of replicating in chimeric mice harboring replaced human hepatocyte cells when exactly the same amount of the G1, G3 and G4 HEV suspension is used (manuscript in preparation). These results indicate that the human hepatocyte is a major target cell for HEV infection, and HEV is not capable of replicating in mice.

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REFERENCES

1. Balayan, M. S., Andjaparidze, A. G., Savinskaya, S. S., Ketiladze, E. S., Braginsky, D. M., Savinov, A. P. and Poleschuk, V. F. 1983. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. *Intervirology* **20**: 23–31.
2. Emerson, S. U., Arankalle, A. D., X.J. A, Purdy, M, Schlauder, G.G. and Tsarev, S.A. 2005. Hepevirus pp. 853–857. *In*: Fauquet, M. A. M. C.M., Maniloff, J., Desselberger, U.and Ball, L.A. Virus Taxonomy, VIIIth Report of the ICTV. (eds.), Elsevier/Academic Press; , London.
3. Emerson, S. U. and Purcell, R. H. 2003. Hepatitis E virus. *Rev. Med. Virol.* **13**: 145–154.
4. Favorov, M. O., Kosoy, M. Y., Tsarev, S. A., Childs, J. E. and Margolis, H. S. 2000. Prevalence of antibody to hepatitis E virus among rodents in the United States. *J. Infect. Dis.* **181**: 449–455.
5. Hussaini, S. H., Skidmore, S. J., Richardson, P., Sherratt, L. M., Cooper, B. T. and O'Grady, J. G. 1997. Severe hepatitis E infection during pregnancy. *J. Viral. Hepat.* **4**:51–54.
6. Jameel, S. 1999. Molecular biology and pathogenesis of hepatitis E virus. *Expert. Rev. Mol. Med.* **1999**: 1–16.
7. Kuno, A., Ido, K., Isoda, N., Satoh, Y., Ono, K., Satoh, S., Inamori, H., Sugano, K., Kanai, N., Nishizawa, T. and Okamoto, H. 2003. Sporadic acute hepatitis E of a 47-year-old man whose pet cat was positive for antibody to hepatitis E virus. *Hepatol. Res.* **26**: 237–242.
8. Li, T. C., Chijiwa, K., Sera, N., Ishibashi, T., Etoh, Y., Shinohara, Y., Kurata, Y., Ishida, M., Sakamoto, S., Takeda, N. and Miyamura, T. 2005. Hepatitis E virus transmission from wild boar meat. *Emerg. Infect. Dis.* **11**:1958–1960.
9. Li, T. C., Saito, M., Ogura, G., Ishibashi, O., Miyamura, T. and Takeda, N. 2006. Serologic evidence for hepatitis E virus infection in mongoose. *Am. J. Trop. Med. Hyg.* **74**: 932-936.
10. Li, T. C., Suzaki, Y., Ami, Y., Dhole, T. N., Miyamura, T. and Takeda, N. 2004. Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus-like particles. *Vaccine* **22**: 370–377.
11. Li, T. C., Zhang, J., Shinzawa, H., Ishibashi, M., Sata, M., Mast, E. E., Kim, K., Miyamura, T. and Takeda, N. 2000. Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. *J. Med. Virol.* **62**: 327–333.
12. Matsuura, Y., Suzuki, M., Yoshimatsu, K., Arikawa, J., Takashima, I., Yokoyama, M., Igota, H., Yamauchi, K., Ishida, S., Fukui, D., Bando, G., Kosuge, M., Tsunemitsu, H., Koshimoto, C., Sakae, K., Chikahira, M., Ogawa, S., Miyamura, T., Takeda, N. and Li, T. C. 2007. Prevalence of antibody to hepatitis E virus among wild sika deer, Cervus nippon, in Japan. *Arch. Virol.* **152**: 1375–1381.
13. McCaustland, K. A., Krawczynski, K., Ebert, J. W., Balayan, M. S., Andjaparidze, A. G., Spelbring, J. E., Cook, E. H., Humphrey, C., Yarbough, P. O., Favorov, M. O., Carson, D., Bradley, D. W. and Robertson, B. H. 2000. Hepatitis E virus infection in chimpanzees: a retrospective analysis. *Arch. Virol.* **145**: 1909–1918.
14. Meng, X. J. 2000. Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? *J. Hepatol.* **33**: 842–845.
15. Meng, X. J., Dea, S., Engle, R. E., Friendship, R., Lyoo, Y. S., Sirinarumitr, T., Urairong, K., Wang, D., Wong, D., Yoo, D., Zhang, Y., Purcell, R. H. and Emerson, S. U. 1999. Prevalence of antibodies to the hepatitis E virus in pigs from countries where hepatitis E is common or is rare in the human population. *J. Med. Virol.* **59**: 297–302.
16. Meng, X. J., Halbur, P. G., Haynes, J. S., Tsareva, T. S., Bruna, J. D., Royer, R. L., Purcell, R. H. and Emerson, S. U. 1998. Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV. *Arch. Virol.* **143**: 1405–1415.
17. Meng, X. J., Halbur, P. G., Shapiro, M. S., Govindarajan, S., Bruna, J. D., Mushahwar, I. K., Purcell, R. H. and Emerson, S. U. 1998. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. *J. Virol.* **72**: 9714–9721.
18. Meng, X. J., Purcell, R. H., Halbur, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S., Haynes, J. S., Thacker, B. J. and Emerson, S. U. 1997. A novel virus in swine is closely related to the human hepatitis E virus. *Proc. Natl. Acad. Sci. U.S.A.* **94**: 9860–9865.
19. Mochizuki, M., Ouchi, A., Kawakami, K., Ishida, T., Li, T. C., Takeda, N., Ikeda, H. and Tsunemitsu, H. 2006. Epidemiological study of hepatitis E virus infection of dogs and cats in Japan. *Vet. Rec.* **159**: 853–854.
20. Nakai, I., Kato, K., Miyazaki, A., Yoshii, M., Li, T. C., Takeda, N., Tsunemitsu, H. and Ikeda, H. 2006. Different fecal shedding patterns of two common strains of hepatitis E virus at three Japanese swine farms. *Am. J. Trop. Med. Hyg.* **75**: 1171–1177.
21. Nakamura, M., Takahashi, K., Taira, K., Taira, M., Ohno, A., Sakugawa, H., Arai, M. and Mishiro, S. 2006. Hepatitis E virus infection in wild mongooses of Okinawa, Japan: Demonstration of anti-HEV antibodies and a full-genome nucleotide sequence. *Hepatol. Res.* **34**: 137–140.
22. Nanda, S. K., Panda, S. K., Durgapal, H. and Jameel, S. 1994. Detection of the negative strand of hepatitis E virus RNA in the livers of experimentally infected rhesus monkeys: evidence for viral replication. *J. Med. Virol.* **42**: 237–240.
23. Nishizawa, T., Takahashi, M., Endo, K., Fujiwara, S., Sakuma, N., Kawazuma, F., Sakamoto, H., Sato, Y., Bando, M. and Okamoto, H. 2005. Analysis of the full-length genome of hepatitis E virus isolates obtained from wild boars in Japan. *J. Gen. Virol.* **86**: 3321–3326.
24. Panda, S. K., Thakral, D. and Rehman, S. 2007. Hepatitis E virus. *Rev. Med. Virol.* **17**: 151–180.
25. Purcell, R. H. 1996. Hepatitis E virus, pp. 2831–2843. *In*: Fields, K. D. and Howley, P.M. eds., Fields Virology, 3rd. Lippincott - Raven, Philadelphia.
26. Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F. and Okamoto, H. 2003. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. *J. Gen. Virol.* **84**: 851–862.
27. Takahashi, M., Nishizawa, T. and Okamoto, H. 2003. Identification of a genotype III swine hepatitis E virus that was isolated from a Japanese pig born in 1990 and that is most closely related to Japanese isolates of human hepatitis E virus. *J. Clin. Microbiol.* **41**: 1342–1343.
28. Tei, S., Kitajima, N., Takahashi, K. and Mishiro, S. 2003. Zoonotic transmission of hepatitis E virus from deer to human

beings. *Lancet* **362**: 371–373.

29. Tsarev, S. A., Emerson, S. U., Tsareva, T. S., Yarbough, P. O., Lewis, M., Govindarajan, S., Reyes, G. R., Shapiro, M. and Purcell, R. H. 1993. Variation in course of hepatitis E in experimentally infected cynomolgus monkeys. *J. Infect. Dis.* **167**: 1302–1306.

30. Watanabe, T., Katagiri, J., Kojima, H., Kamimura, T., Ichida, F., Ashida, M., Hamada, C. and Shibayama, T. 1987. Studies on transmission of human non-A, non-B hepatitis to marmosets. *J. Med. Virol.* **22**: 143–156.

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BRIEF REPORT

Efficient production of type 2 porcine circovirus-like particles by a recombinant baculovirus

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Abstract The capsid protein of PCV2 was expressed by using a recombinant baculovirus with insect Tn5 cells. A large amount of 28-kDa protein was released into the culture medium and self-assembled into PCV2-like particles (PCV2-LPs) with a buoyant density of 1.365 g/cm^3 and a diameter of 20 nm. PCV2-LPs were efficiently expressed, yielding 1 mg of purified particles per 10^7 Tn5 cells. The PCV2-LPs have antigenicity similar to that of authentic PCV2 particles, allowing us to develop a method for sensitively detecting PCV2-specific IgG antibodies. In addition, the PCV2-LPs appeared to be the most promising PCV2 vaccine candidate, by virtue of their potent immunogenicity.

Postweaning multisystemic wasting syndrome (PMWS) is a disease with low morbidity but high mortality in swine. PMWS has a serious economic impact on the global swine industry. It was first reported in western Canada in 1991 and later identified in the United States, Mexico, Europe, and Asia [2]. Porcine circovirus type 2 (PCV2) is the primary causative agent of PMWS, though the etiology of this disease has not been fully elucidated. The isolation of viruses from tissues of affected swine led to the identification of PCV2 [5–8, 20]. PCV2, a member of the family *Circoviridae* [25], is a small spherical nonenveloped virus with a single-stranded closed circular genomic DNA of 1.7 kb in length [1]. Two major open reading frames (ORFs) oriented in opposite directions have been identified. ORF1 encodes two proteins: Rep and its truncated form, Rep′, both of which are essential for viral DNA replication. ORF2 encodes a major structural protein that has type-specific epitopes [17, 22] and is very immunogenic and strongly associated with the induction of neutralizing antibodies [24], suggesting its potential use in diagnostic assays as well as vaccine development. In the present study, we expressed the PCV2 capsid protein by using a recombinant baculovirus in Tn5 insect cells. The capsid protein self-assembled into PCV2-like particles (PCV2-LPs) and was released into the culture medium. The PCV2-LPs possess similar antigenicity to that of the native PCV2 particles and appear to be a good antigen for the sensitive detection of PCV2-specific antibodies. Our study also demonstrated that PCV2-LPs are the most promising PCV2 vaccine candidate.

Viral DNA was extracted from the PCV2 Yamagata strain [23], and the full-length ORF2 of PCV2 (PCV2-ORF2) was amplified by PCR with forward primer PCV2-D1 (5′-AA**GGATCC**ATGACGTATCCAAGGAGGCGTT-3′) and reverse primer PCV2-U1 (5′-GC**TCTAGA**TTAGGGT TTAAGTGGGGGGTCT-3′). The forward primer contained the *Bam*HI site before the start codon, and the reverse primer

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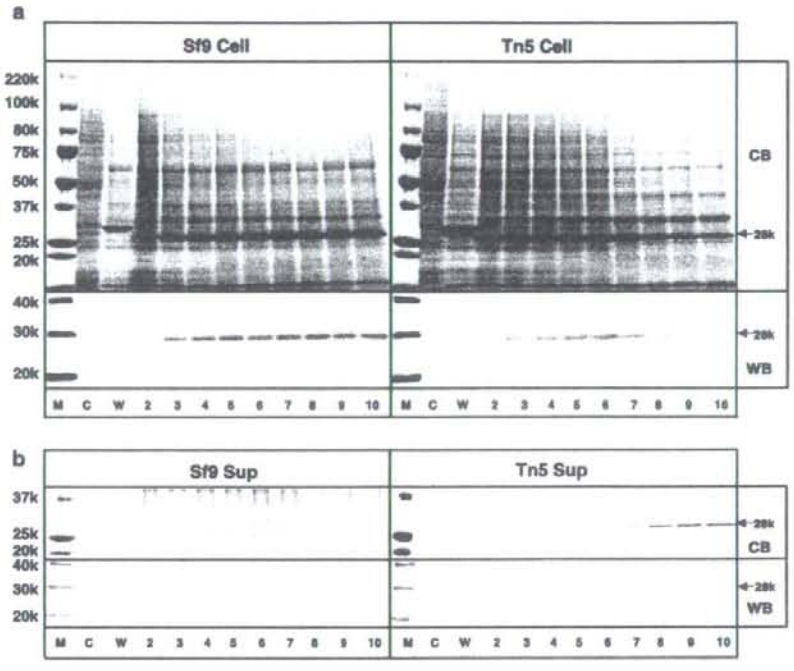

Fig. 1 Time course of the expression of PCV2 capsid protein in insect cells. Insect Sf9 and Tn5 cells were infected with the recombinant baculovirus AcPCV2-ORF2, incubated at 26.5°C, and harvested on the indicated days (2–10 days). Five microliters of the culture medium and the lysate from 10^5 cells were analyzed by SDS-PAGE. Protein bands were visualized by Coomassie blue staining (*CB*) or by Western blot assay with anti-PCV2 rabbit serum (*WB*). The cell lysate (a) and culture medium (b) were analyzed separately. *M* molecular weight marker, *C* uninfected cell, *W* wild-type baculovirus-infected cells, *lanes* 2–10, 2–10 days p.i

contained the *Xba*I site after the stop codon. The amplified ORF2 fragment was purified by using a gel extraction kit (Qiagen, Valencia, CA) and was first digested with *Bam*HI and then partially digested with *Xba*I. The purified 700-bp fragment was ligated into transfer vector pVL1393 (Pharmingen, San Diego, CA) by a ligation kit (Takara, Shiga, Japan), and a transfer plasmid pVL1393/PCV2-ORF2 was constructed.

A recombinant baculovirus was constructed and capsid proteins were expressed as previously described [14, 16]. Insect Sf9 and Tn5 cells were infected with a recombinant baculovirus, AcPCV2-ORF2, containing the entire PCV2 capsid protein. The infected cells were harvested daily until 10 days p.i. The proteins expressed in infected cells were analyzed by SDS-PAGE followed by Coomassie blue staining and by Western blot assay using a rabbit anti-PCV2 antibody (Fig. 1). A major band with a molecular mass of 28 kDa was observed in cell lysate of both Sf9 and Tn5 cells (Fig. 1a). The 28-kDa protein was first detected on day 2 in both Sf9 and Tn5 cells, and peaked on day 5 p.i. This 28-kDa protein was detected on day 4 p.i. in the supernatant of Tn5 cells and increased until day 10 p.i. (Fig. 1b), whereas it was not detected in the supernatant of Sf9 cells.

The culture medium of the AcPCV2-ORF2-infected Tn5 cells was harvested at 7 days p.i., and the PCV2 capsid protein was purified by CsCl gradient centrifugation. The 28-kDa protein appeared mainly in fractions 3, 4, and 5, which had an average density of 1.365 g/ml[3] (Fig. 2a). Examination of these fractions by electron microscopy revealed spherical particles with diameters of ~20 nm. The morphology of these particles was similar to that of the authentic PCV2 particles (Fig. 2b), indicating that the 28-kDa protein formed virus-like particles (VLPs) (PCV2-LPs). The yield of the purified VLPs was 1 mg per 10^7 Tn5 cells in culture medium. We tried to purify the PCV2 capsid protein from the infected Sf9 cells, but only a few PCV2-LPs were obtained from the cell lysate, and no VLPs were obtained from the supernatant (data not shown).

An ELISA to detect antibodies was developed by using PCV2-LPs as an antigen. This antibody ELISA showed a low background, probably because the PCV2-LPs were highly purified. The cutoff value of IgG was determined by using 30 serum samples from wild boar that were negative for anti-PCV2 IgG by Western blot assay (data not shown). The OD values of these sera were between 0.036 and 0.249, and the mean value was 0.062 with a standard deviation (SD) of 0.046. Therefore, the cutoff value, the mean value+3SD, was calculated as 0.200. The PCV2-specific IgG elicited in swine experimentally infected with the PCV2 Yamagata strain was detected by the antibody ELISA. As depicted in Fig. 3a, significant IgG antibody titers were observed, indicating that the purified PCV2-LPs had similar antigenicity to native PCV2. A panel of 105

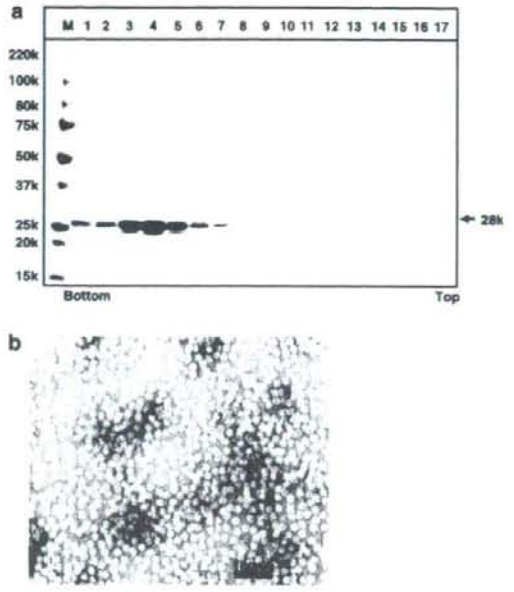

Fig. 2 **a** Purification PCV2-LPs by CsCl gradient centrifugation. The supernatant of the recombinant baculovirus-infected Tn5 cells was centrifuged for 3 h at 31,000 rpm in a Beckman SW32Ti rotor. The pellet was resuspended in 4.5 ml ExCell 405 and mixed with 2.1 g CsCl, then centrifuged for 24 h at 35,000 rpm in an SW55Ti rotor. Aliquots from the gradient were analyzed by electrophoresis on a 5–20% polyacrylamide gel and stained with Coomassie blue. The bottom and top of the gradient and the positions of molecular weight standards are indicated. **b** Electron microscopy of PCV2-LPs. Purified PCV2-LPs were stained with 2% uranyl acetate and observed by EM. *Bar* 100 nm

swine serum samples was collected from a slaughterhouse in Japan in 2006. All of the serum samples were diluted 1:200 for the ELISA test. The result showed that all sera from healthy slaughtered domestic pigs were positive for IgG against PCV2, and the OD values were above 0.65 without exception (data not shown).

Antibodies to the PCV2-LPs were prepared in rabbits and guinea pigs by subcutaneous injection of the purified PCV2-LPs. After being injected two times, the animals produced high levels of IgG antibodies, and the titers reached levels as high as 1:1,638,400 in the antibody ELISA. Immunogenicity of the VLPs was examined using an ELISA with rabbit and guinea pig hyperimmune sera as the capture and detector antibodies, respectively. As shown in Fig. 3b, the sensitivity reached 0.16 ng/ml of PCV2-LPs when a cutoff OD value of 0.2 was used, and native PCV2 particles were detected in the culture medium of PCV2-infected PK-15 cells, yielding an antigen titer of 1:32. Native PCV2 particles concentrated by centrifugation ($100{,}000\times g$, 2 h) showed an increased titer of 1:256 (data not shown). These results demonstrated that the PCV2-LPs were immunogenic and able to elicit antibodies capable of binding to native PCV2 particles.

PCV2 is a pivotal causative agent of PMWS and is recognized as a major economic problem in the porcine industry worldwide [2, 10, 12, 26]. In addition, PCV2 infection is subclinical in some swine, and these swine become carriers and cause longer virus circulation in herds. However, the development of a vaccine and diagnosis are hampered by a low yield of PCV2 in cell culture. PCV2 capsid protein is the major structural protein and is highly

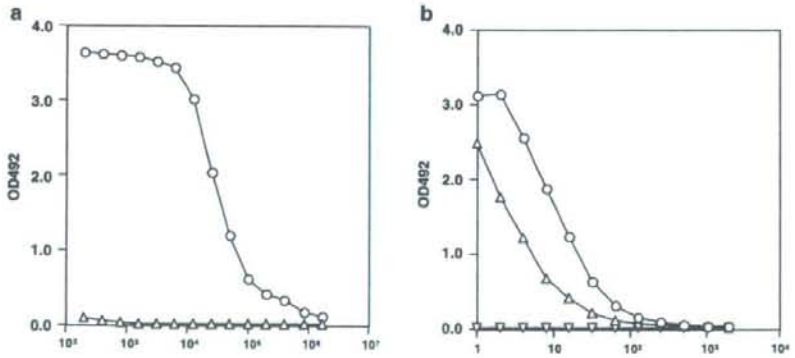

Fig. 3 **a** Antigenicity of PCV2-LPs. A swine was experimentally infected. The pre-serum was obtained before PCV2 infection, and the post-serum was obtained 4 weeks after infection. Both pre- (*open triangle*) and post-sera (*open circle*) were examined for IgG antibody by twofold dilutions starting from 1:200. **b** Immunogenicity of PCV2-LPs. Anti-PCV2-LP serum from a rabbit was used to coat a 96-well microplate. The binding of recombinant PCV2-LPs (10 ng/ml) (*open circle*) and native PCV2 particles produced in the supernatant of Yamagata-strain-infected PK-15 cells (*open triangle*) was examined by antigen ELISA. The OD value was considered positive when the absorbance was ≥ 0.150. The culture medium from uninfected PK-15 cells (*open inverted triangle*) was used as a negative control

immunogenic. As both neutralizing monoclonal antibodies and neutralizing swine sera have been shown to react with the capsid protein [13, 19, 24], the capsid protein is an attractive immunogen for vaccine development and diagnosis.

The recombinant baculovirus expression system has long been used to express proteins as well as to generate VLPs in various DNA and RNA viruses [3, 11, 14, 15, 18]. This system has many advantages over other systems: efficient expression, proper post-translational modification, correct conformation, and self-assembly of the capsid protein into VLPs, which usually retain the immunogenicity as well as physicochemical properties of their native virions. Sf9 and Tn5 cells are commonly used cell lines for the baculovirus expression system. Previous studies have shown that PCV2 capsid protein expressed in Sf9 cells self-assembled into VLPs [22]. However, although VLPs were generated in the Sf9 cells, they were not released into the culture medium. When we expressed the PCV2 capsid in Sf9 cells, the same result was observed. In contrast, when the recombinant PCV2 capsid protein was expressed in Tn5 cells, the capsid protein efficiently self-assembled into VLPs and, interestingly, was released into the culture medium, making purification of the VLPs easy. The yield of the purified VLPs reached 1 mg per 10^7 Tn5 cells, providing a virtually unlimited supply of highly purified PCV2-LPs.

The feasibility of the capsid protein-based PCV2 vaccine was demonstrated in several previous studies, where the successful induction of specific serum antibodies was observed [4, 9, 27]. It was also found that the absence of PCV2-neutralizing antibodies is well correlated with the virus replication and development of PMWS [21], indicating that the humoral immune response plays an important role in the prophylaxis of PCV2 infection. There are several advantages to using PCV2-LPs as a vaccine: First, the PCV2-LPs have an excellent safety profile, since there is no viral genome in the particles. Second, the PCV2-LPs have antigenicity and immunogenicity similar to those of the native PCV2. Third, the PCV2-LPs are morphologically the same as the native PCV2, and the conformational antigenic epitopes may be properly retained. Therefore, the PCV2-LPs share the same immunogenicity with the native PCV2 virion. In fact, antibodies elicited with PCV2-LPs strongly reacted with the native virion. These results clearly demonstrate that PCV2-LPs are a promising PCV2 vaccine candidate.

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References

1. Allan GM, Ellis JA (2000) Porcine circoviruses: a review. J Vet Diagn Invest 12:3–14
2. Allan GM, Mc Neilly F, Meehan BM, Kennedy S, Mackie DP, Ellis JA, Clark EG, Espuna E, Saubi N, Riera P, Botner A, Charreyre CE (1999) Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. Vet Microbiol 66:115–123
3. Baumert TF, Vergalla J, Satoi J, Thomson M, Lechmann M, Herion D, Greenberg HB, Ito S, Liang TJ (1999) Hepatitis C virus-like particles synthesized in insect cells as a potential vaccine candidate. Gastroenterology 117:1397–1407
4. Blanchard P, Mahe D, Cariolet R, Keranflec'h A, Baudouard MA, Cordioli P, Albina E, Jestin A (2003) Protection of swine against post-weaning multisystemic wasting syndrome (PMWS) by porcine circovirus type 2 (PCV2) proteins. Vaccine 21:4565–4575
5. Ellis J, Hassard L, Clark E, Harding J, Allan G, Willson P, Strokappe J, Martin K, McNeilly F, Meehan B, Todd D, Haines D (1998) Isolation of circovirus from lesions of pigs with post-weaning multisystemic wasting syndrome. Can Vet J 39:44–51
6. Ellis J, Krakowka S, Lairmore M, Haines D, Bratanich A, Clark E, Allan G, Konoby C, Hassard L, Meehan B, Martin K, Harding J, Kennedy S, McNeilly F (1999) Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. J Vet Diagn Invest 11:3–14
7. Ellis JA, Bratanich A, Clark EG, Allan G, Meehan B, Haines DM, Harding J, West KH, Krakowka S, Konoby C, Hassard L, Martin K, McNeilly F (2000) Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired post-weaning multisystemic wasting syndrome. J Vet Diagn Invest 12:21–27
8. Fenaux M, Halbur PG, Gill M, Toth TE, Meng XJ (2000) Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J Clin Microbiol 38:2494–2503
9. Fenaux M, Opriessnig T, Halbur PG, Elvinger F, Meng XJ (2004) A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J Virol 78:6297–6303
10. Hasslung F, Wallgren P, Ladekjaer-Hansen AS, Botner A, Nielsen J, Wattrang E, Allan GM, McNeilly F, Ellis J, Timmusk S, Belak K, Segall T, Melin L, Berg M, Fossum C (2005) Experimental reproduction of postweaning multisystemic wasting syndrome (PMWS) in pigs in Sweden and Denmark with a Swedish isolate of porcine circovirus type 2. Vet Microbiol 106:49–60
11. Ishii Y, Ozaki S, Tanaka K, Kanda T (2005) Human papillomavirus 16 minor capsid protein L2 helps capsomeres assemble independently of intercapsomeric disulfide bonding. Virus Genes 31:321–328
12. Kim J, Chung HK, Jung T, Cho WS, Choi C, Chae C (2002) Postweaning multisystemic wasting syndrome of pigs in Korea: prevalence, microscopic lesions and coexisting microorganisms. J Vet Med Sci 64:57–62
13. Lekcharoensuk P, Morozov I, Paul PS, Thangthumniyom N, Wajjawalku W, Meng XJ (2004) Epitope mapping of the major

capsid protein of type 2 porcine circovirus (PCV2) by using chimeric PCV1 and PCV2. J Virol 78:8135–8145
14. Li TC, Yamakawa Y, Suzuki K, Tatsumi M, Razak MA, Uchida T, Takeda N, Miyamura T (1997) Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol 71:7207–7213
15. Li TC, Takeda N, Kato K, Nilsson J, Xing L, Haag L, Cheng RH, Miyamura T (2003) Characterization of self-assembled virus-like particles of human polyomavirus BK generated by recombinant baculoviruses. Virology 311:115–124
16. Li TC, Takeda N, Miyamura T, Matsuura Y, Wang JC, Engvall H, Hammar L, Xing L, Cheng RH (2005) Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus. J Virol 79:12999–13006
17. Mahe D, Blanchard P, Truong C, Arnauld C, Le Cann P, Cariolet R, Madec F, Albina E, Jestin A (2000) Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes. J Gen Virol 81:1815–1824
18. Matsuo E, Tani H, Lim C, Komoda Y, Okamoto T, Miyamoto H, Moriishi K, Yagi S, Patel AH, Miyamura T, Matsuura Y (2006) Characterization of HCV-like particles produced in a human hepatoma cell line by a recombinant baculovirus. Biochem Biophys Res Commun 340:200–208
19. McNeilly F, McNair I, Mackie DP, Meehan BM, Kennedy S, Moffett D, Ellis J, Krakowka S, Allan GM (2001) Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. Arch Virol 146:909–922
20. Meehan BM, McNeilly F, Todd D, Kennedy S, Jewhurst VA, Ellis JA, Hassard LE, Clark EG, Haines DM, Allan GM (1998) Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J Gen Virol 79(Pt 9):2171–2179
21. Meerts P, Misinzo G, Lefebvre D, Nielsen J, Botner A, Kristensen CS, Nauwynck HJ (2006) Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Vet Res 2:6
22. Nawagitgul P, Morozov I, Bolin SR, Harms PA, Sorden SD, Paul PS (2000) Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J Gen Virol 81:2281–2287
23. Onuki A, Abe K, Togashi K, Kawashima K, Taneichi A, Tsunemitsu H (1999) Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. J Vet Med Sci 61:1119–1123
24. Pogranichnyy RM, Yoon KJ, Harms PA, Swenson SL, Zimmerman JJ, Sorden SD (2000) Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunol 13:143–153
25. Pringle CR (1999) Virus taxonomy-1999. The universal system of virus taxonomy, updated to include the new proposals ratified by the International Committee on Taxonomy of Viruses during 1998. Arch Virol 144:421–429
26. Rodriguez-Arrioja GM, Segales J, Calsamiglia M, Resendes AR, Balasch M, Plana-Duran J, Casal J, Domingo M (2002) Dynamics of porcine circovirus type 2 infection in a herd of pigs with postweaning multisystemic wasting syndrome. Am J Vet Res 63:354–357
27. Wang X, Jiang P, Li Y, Jiang W, Dong X (2007) Protection of pigs against post-weaning multisystemic wasting syndrome by a recombinant adenovirus expressing the capsid protein of porcine circovirus type 2. Vet Microbiol 121:215–224

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ORIGINAL ARTICLE

Production of monoclonal antibodies against hepatitis E virus capsid protein and evaluation of their neutralizing activity in a cell culture system

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Abstract Nine murine monoclonal antibodies (mAbs) generated against a recombinant ORF2 protein (amino acids 111–660) of a genotype 4 hepatitis E virus (HEV) strain recognized four sets of epitopes by pairwise competitive ELISA. One mAb (H6225) was able to capture HEV efficiently regardless of genotype and was tested for its ability to neutralize a genotype 3 HEV strain (JE03-1760F) in a recently developed cell culture system for HEV in a hepatocarcinoma cell line (PLC/PRF/5). When PLC/PRF/5 cells were inoculated with HEV (4.0×10^5 or 4.0×10^6 copies/ml) incubated with 100 μg/ml of a negative control mAb, HEV RNA in the culture medium continued to be detectable after day 14 or 12 post-inoculation (dpi), respectively. However, when cells were inoculated with the two distinct concentrations of HEV that had been mixed with 100 μg/ml of H6225, the harvested culture supernatants were negative for HEV RNA throughout the 60-day observation period. Upon prior mixing of the virus with 10 μg/ml of H6225, HEV RNA in culture supernatant continued to be undetectable until 46 or 28 dpi, respectively. In conclusion, one mAb (H6225) against HEV capsid protein that can efficiently neutralize HEV in vitro was obtained in the present study.

The nucleotide sequence data reported in this study have been assigned Genbank/EMBL/DDBJ accession numbers AB360347-AB360348.

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Introduction

Hepatitis E virus (HEV) causes acute hepatitis E in humans, which is a major public health concern in many developing countries of Asia, Africa and Latin America [33]. Sporadic cases of hepatitis E that are not related to travel to endemic areas have also been identified in numerous industrialized countries including the United States, European countries and Japan [1, 5, 10, 17, 21, 28, 32]. Accumulating lines of evidence indicate that hepatitis E is a zoonosis [5, 25–27, 29, 31, 38, 47, 51]. HEV infection runs an acute course, normally resulting in resolution within a few weeks after onset, with mortality ranging from 0.5 to 1% among the general population to as high as 20% among infected pregnant women [16, 33]. The presence of a chronic or persistent HEV infection, however, has not been described.

HEV is a single-stranded, positive-sense RNA virus with an approximately 7.2 kb genome packaged within a non-enveloped capsid and is classified as the sole member of the genus *Hepevirus* [3]. Its genome contains a short 5′ untranslated region (UTR) followed by three open reading frames (ORFs: ORF1, ORF2 and ORF3), and then a short 3′ UTR with a poly(A) tail [42]. Extensive genomic diversity has been noted among HEV isolates, and HEV sequences have been classified into four genotypes, represented by the Burmese strain (genotype 1), Mexican strain (genotype 2), US strain (genotype 3) and the new Chinese strain (genotype 4). Genotype 1 is responsible for the majority of HEV infections in developing countries in Asia

and Africa; genotype 2 consists of strains not only in Mexico but also in African countries including Chad, Namibia and Nigeria; genotype 3 is widely distributed throughout the world except in Africa; and genotype 4 is distributed exclusively in Asian countries [3, 20, 30, 35].

The capsid protein encoded by ORF2 is the most immunogenic protein expressed by HEV and is responsible for the induction of protective humoral immune responses. A major neutralizing epitope was identified on the ORF2 capsid protein of HEV [36, 37], and some monoclonal antibodies (mAbs) raised against the genotype 1 capsid protein showed neutralizing activity [6, 34, 36, 52]. In the present study, mAbs against a recombinant genotype 4 ORF2 protein that had been expressed in the pupae of silkworm were generated and characterized, and their abilities to bind recombinant ORF2 proteins of genotype 1, 3, or 4 and native HEV particles in feces from patients infected with HEV of genotype 1, 3, or 4 were evaluated. Furthermore, one mAb that was capable of efficiently binding native HEV particles was assessed for its ability to neutralize HEV in a recently developed cell culture system [46].

Materials and methods

Preparation and purification of recombinant HEV ORF2 proteins

Recombinant HEV ORF2 proteins of genotypes 1 and 3 (rHEV ORF2-G1 and rHEV ORF2-G3, respectively) with their N-terminus truncated [amino acid (aa) residues 111–660 of ORF2], were expressed by a recombinant baculovirus according to the method described for the rHEV ORF2-G4 protein (HE-J1 strain) by Mizuo et al. [28]. Briefly, the putative capsid gene (ORF2) of an HEV isolate of genotype 1 (Ne-A312 strain: accession no. AB360347) was amplified by reverse transcription (RT)-polymerase chain reaction (PCR) with the following set of primers: sense primer, 5′-GGA TCC **ATG** GCG GTC GCC CCG GCC CAT GAC-3′; antisense primer, 5′-GAG CTC **ATC** ATA ACT CCC GAG TTT TAC C-3′. The introduced restriction sites in the primers (*Bam*HI in the sense primer and *Sac*I in the antisense primer) are underlined, and the truncated ORF2 start codon (ATG) and two in-frame stop codons (successive TGA) are indicated in bold type. The putative capsid gene of an HEV isolate of genotype 3 (HE-JA10 strain: accession no. AB360348) was amplified in a similar manner. The respective PCR products were cloned into the pT7BlueT vector (Novagen, Inc., Madison, WI) and digested with *Bam*HI and *Sac*I. The resulting 2-kb fragments were inserted into the *Bgl*II-*Sac*I site of a transfer vector, pYNG (Katakura Industries Co. Ltd., Saitama, Japan), and sequenced by the methods described previously [31]. The 5′-truncated putative capsid gene sequences of genotypes 1 and 3 encoding 550 aa were cloned into a baculovirus expression vector and expressed in the pupae of silkworm.

The silkworm pupae were lysed in 20 mM PIPES [piperazine-*N*,*N*′-bis (2-hydroxypropane-3-sulfonic acid)] buffer (pH 6.6) containing 10% (vol/vol) glycerol, 0.1 M NaCl, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 10 mM benzamidine, and then homogenated in 1% (wt/vol) Triton X-100, followed by centrifugation at 100,000×*g* at 4°C for 15 min. The resulting supernatant was treated with polyethylenglycol at a final concentration of 4% (wt/vol). The precipitates were redissolved in 20 mM Tris–HCl (pH 8.0), and purified by anion-exchange chromatography. Following purification, the purified protein was shown to produce one predominant band of 61 kDa on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

Production of monoclonal antibodies

mAbs were raised against purified rHEV ORF2-G4 protein by a method described elsewhere [49] with slight modifications. Briefly, BALB/c mice were injected twice intraperitoneally with 30 μg of the rHEV ORF2-G4 protein in complete Freund's adjuvant (DIFCO Laboratories, Detroit, MI) on day 0 and in incomplete adjuvant (DIFCO Laboratories) on day 14, followed by intravenous injection of 15 μg of the same antigen (without adjuvant) on day 29, 3 days before fusion. NS-1 myeloma cells were fused with immunized spleen cells at a ratio of 1:10. The screening of mAbs was done by enzyme-linked immunosorbent assay (ELISA) using the same antigen immobilized on wells of an immunoplate (part no. 762071; Greiner Bio-One GmbH, Frickenhausen, Germany). Bound antibodies were detected by peroxidase-conjugated sheep affinity-purified antibody to mouse IgG or IgM (Cappel/MP Biochemicals, Solon, OH). Hybridomas secreting anti-HEV antibody of desired specificity were propagated in the peritoneal cavity of mice that had been made ascitic by the injection of 2,6,10,14-tetramethylpentadecane. Ascites fluid was harvested approximately 10 days after the implantation, and γ-globulin fractions were precipitated with 2M $(NH_4)_2SO_4$, and then purified by gel filtration in Sephadex G-200 (GE Healthcare UK Ltd., Buckinghamshire, England). mAbs were tested for their immunoglobulin class/subclass using a mouse monoclonal antibody isotyping kit (Bio-Rad Laboratories).

Western blotting

Purified rHEV ORF2-G4 protein suspended in a loading buffer [50 mM Tris–HCl (pH 6.8), 3% (wt/vol) SDS, 0.06% bromophenol blue, 10% (vol/vol) glycerol] was separated into two tubes. One was incubated in the presence of 3% (vol/vol) 2-mercaptoethanol in boiling water for 5 min, while the other was left untreated. The denatured and non-denatured samples were subjected to SDS-PAGE in a 12% acrylamide gel, followed by transfer onto a nitrocellulose filter membrane (Hybond-ECL, GE Healthcare). The membrane was immersed in Tris-buffered saline with Tween-20 (TBST) [10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 0.1% (vol/vol) Tween-20] containing 5% of ECL blocking agent (GE Healthcare) and, after being washed with TBST, incubated at room temperature for 1 h with 10 μg/ml of each of the mAbs raised in the present study as the primary antibody. After being washed, the membrane was incubated with ECL anti-mouse IgG or IgM, horseradish peroxidase-linked species-specific whole antibody from sheep (1:2,500; GE Healthcare) and then examined using a chemical luminescence system (ECL Western blotting detection reagents and analysis system; GE Healthcare). ECL DualVue Western Blotting Markers (GE Healthcare) were used as protein markers.

ELISA for determination of binding with recombinant HEV ORF2 proteins of various genotypes

This ELISA was performed according to a previously described method [28] with some modifications. Briefly, wells of microplates were coated with 100 μl of the recombinant ORF2 proteins [rHEV ORF2-G1, -G3, and -G4: 5 μg/ml in 10 mM phosphate-buffered saline, pH 7.5 (PBS)] and incubated at 25°C for 18 h. Two hundred microliters of PBS containing 0.1% (wt/vol) bovine serum albumin (BSA; Oriental Yeast Co., Ltd., Tokyo, Japan) (PBS-BSA) was added. The microplates were incubated at room temperature for 2 h with shaking. The blocking buffer was discarded, and each well was washed with saline containing 0.05% (vol/vol) Tween-20 (Bio-Rad Laboratories). One hundred microliters each of mAbs (0.003–10 μg/ml) diluted with PBS-BSA was added to each well. The microplates were incubated at room temperature for 1 h with gentle agitation and were then washed with washing solution (0.05% Tween-20 in saline). One hundred microliters of PBS containing 25% (vol/vol) fetal bovine serum (Sigma-Aldrich, St Louis, MO) and peroxidase-conjugated goat IgG fraction to mouse IgG (whole molecule) (Cappel/MP Biochemicals) or peroxidase-conjugated affinity purified anti-mouse IgMμ [mu-chain-specific (goat)] (Rockland Immunochemicals, Inc., Gilbertsville, PA) was added to each well. The microplates were incubated at room temperature for 1 h with gentle agitation and then washed. One hundred microliters of tetramethylbenzidine (TMB) soluble reagent (BioFX Laboratories, Inc., Owings Mills, MD) as a substrate was added to each well. The plate was incubated at room temperature for 30 min in the dark, and then 100 μl of TMB stop buffer (BioFX Laboratories, Inc.) was added to each well. The optical density (OD) of each sample was read at 450 nm.

Biotinylation of mAbs and competitive ELISA

One milliliter of PBS containing mAb (2 mg/ml) was mixed with 27 μl of 10 mM Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL), with a molecular ratio of IgG to biotin of 1:20, and incubated on ice for 2.5 h. The biotinylated mAbs were purified by gel filtration using a PD-10 column (GE Healthcare) and aliquoted and stored at −20°C until assay.

To evaluate the spatial relationships of epitopes recognized by each of the mAbs, pairwise competitive ELISA was performed. Wells of microplates were coated with 100 μl of rHEV ORF2-G4 protein (5 μg/ml in PBS) and incubated at 25°C overnight. After removal of the coating buffer, 200 μl of PBS-BSA was added. The microplates were incubated with gentle shaking at room temperature for 2 h. The blocking buffer was discarded, and each well was washed five times with washing solution (saline with 0.05% Tween-20). Premixtures of biotinylated mAb (final concentration of 10 μg/ml) and unlabeled mAb (final concentration of 0.3, 1, 3, 10, 30, 100, or 300 μg/ml) were prepared in PBS-BSA, and a 100 μl portion was added to each well. An mAb (No. 905) directed against hepatitis B e antigen [39] was included as a negative control of competitors. The microplates were incubated with shaking at room temperature for 1 h and were then washed five times with washing solution. A total of 100 μl of PBS containing 25% (vol/vol) fetal bovine serum and peroxidase-conjugated streptavidin (Pierce Biotechnology) was added to each well. The microplates were incubated with shaking at room temperature for 1 h and then washed five times with washing solution. Then, 100 μl of TMB soluble reagent as a substrate was added to each well. The plate was incubated at room temperature for 30 min in the dark, and then 100 μl of TMB stop buffer was added to each well. The OD value of each well was read at 450 nm. Maximum binding was determined when biotinylated mAbs were added without competitors. The percentage of inhibition was calculated using the formula: [1-(OD value of biotinylated mAb plus non-labeled mAb)/(OD value of biotinylated mAb)] × 100.

Binding of mAbs to HEV particles

An immuno-capture RT-PCR method was used to assess the virus-binding ability of mAbs. Briefly, the wells of a Reacti-Bind Streptavidin High Binding Capacity Coated Plate (Pierce Biotechnology) were washed with saline three times, and 100 µl of 1 µg/ml biotinylated mAb in PBS-BSA containing 0.2% Tween-20 was added to each well. The wells were incubated with gentle shaking at room temperature for 1 h and were then washed five times with saline. One hundred microliters of fecal samples containing HEV of genotype 1, 3 or 4 from infected humans was added to each well and incubated at room temperature for 1 h and then at 4°C overnight. The solution in each well was removed, and the wells were washed three times with saline. One hundred fifty microliters of Trizol-LS reagent (Invitrogen, Carlsbad, CA) and 50 µl of distilled water were directly added twice to each well. The RNA was then extracted and subjected to quantitative detection of HEV RNA.

Quantitation of HEV RNA

Quantitation of HEV RNA was performed by real-time detection RT-PCR according to a previously described method [11] with a slight modification. In brief, extracted RNA was subjected to real-time RT-PCR with a Quanti-Tect Probe RT-PCR Kit (Qiagen, Tokyo, Japan), sense primer (5′-GGT GGT TTC TGG GGT GAC-3′), antisense primer (5′-AGG GGT TGG TTG GAT GAA-3′), and a probe consisting of an oligonucleotide with a 5′-reporter dye (FAM) and a 3′-quencher dye (TAMRA) (5′-FAM-TGA TTC TCA GCC CTT CGC-TAMRA-3′) in a Light-Cycler Apparatus (Roche Diagnostics K.K., Tokyo, Japan). Thermal cycler conditions were 50°C for 20 min at stage 1; 95°C for 15 min at stage 2; and 45 cycles of 95°C for 0 s and 60°C for 60 s at stage 3. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate, and the mean value was adopted.

Neutralization assay in a cell culture system

A hepatocarcinoma cell line (PLC/PRF/5) obtained from American Type Culture Collection (ATCC no. CRL-8024: Manassas, VA) was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; PAA Lab GmbH, Pasching, Austria), 100 U/ml of penicillin G, 100 µg/ml of streptomycin and 2.5 µg/ml of amphotericin B at 37°C in a humidified 5% CO_2 atmosphere, as described previously [46]. For virus infection, confluent cells were trypsinized and diluted 1:4 in medium, and 2 ml was added to wells (diameter of 3.5 cm) of a 6-well microplate (IWAKI, Tsukuba, Japan) 1 or 2 days before virus infection. One hundred microliters of fecal supernatant containing an HEV strain (JE03-1760F) as an inoculum [46] that had been diluted with PBS without Ca^{2+} and Mg^{2+} [PBS(−)] containing 0.2% (wt/vol) BSA (Sigma-Aldrich) and filtrated, was mixed with an equal volume of each of the mAb solutions (2, 20 or 200 µg/ml) and kept at room temperature for 1 h. Monolayers of cultured cells in a 6-well microplate were washed three times with 1 ml of PBS(−), and 0.2 ml of the virus stock mixed with mAb was inoculated on the cells. One hour after inoculation at room temperature, the solution was removed and 2 ml of maintenance medium was added. The maintenance medium used for virus culturing consisted of 50% DMEM and 50% medium 199 (Invitrogen) containing 2% (vol/vol) heat-inactivated FCS and 30 mM $MgCl_2$ at final concentration. Other supplements were the same as in the growth medium. The culture was done at 35.5°C in a humidified 5% CO_2 atmosphere. On the day following inoculation, the inoculated cells were washed five times with 1 ml of PBS(−), and then 2 ml of maintenance medium was added. Then, every other day, one half (1 ml) of the culture medium was replaced with fresh maintenance medium, and the collected medium was stored at −80°C until virus titrations were performed. The HEV load was determined for all or selected series of culture supernatants from the inoculated wells, and representative data were adopted.

Results

Production and characterization of mAbs

Nine hybridoma clones secreting mAbs designated H6206, H6210, H6219, H6222, H6225, H6235, H6242, H6249, and H6253 against rHEV ORF2-G4 were obtained in the present study. Three of these (H6206, H6235, and H6253) were of the IgM class, while the other six were of the IgG1 subclass. All nine mAbs reacted specifically with the rHEV ORF2-G4 protein used as an immunogen as well as two other recombinant proteins of genotypes 1 and 3 (rHEV ORF2-G1 and -G3) in a manner dependent on the concentration of mAbs added as primary antibodies (Fig. 1). At the concentrations of 0.3, 1, 3, and 10 µg/ml, the OD values reacting with the immobilized rHEV ORF2-G1, -G3, or -G4 protein were exclusively >3.0 for all nine mAbs (data not shown for the concentrations of 1, 3, and 10 µg/ml in Fig. 1). Although five mAbs (H6210, H6219, H6235, H6249, and H6253) reacted equally with the three recombinant HEV ORF2 proteins in all mAb concentrations tested, the remaining four mAbs showed genotype-