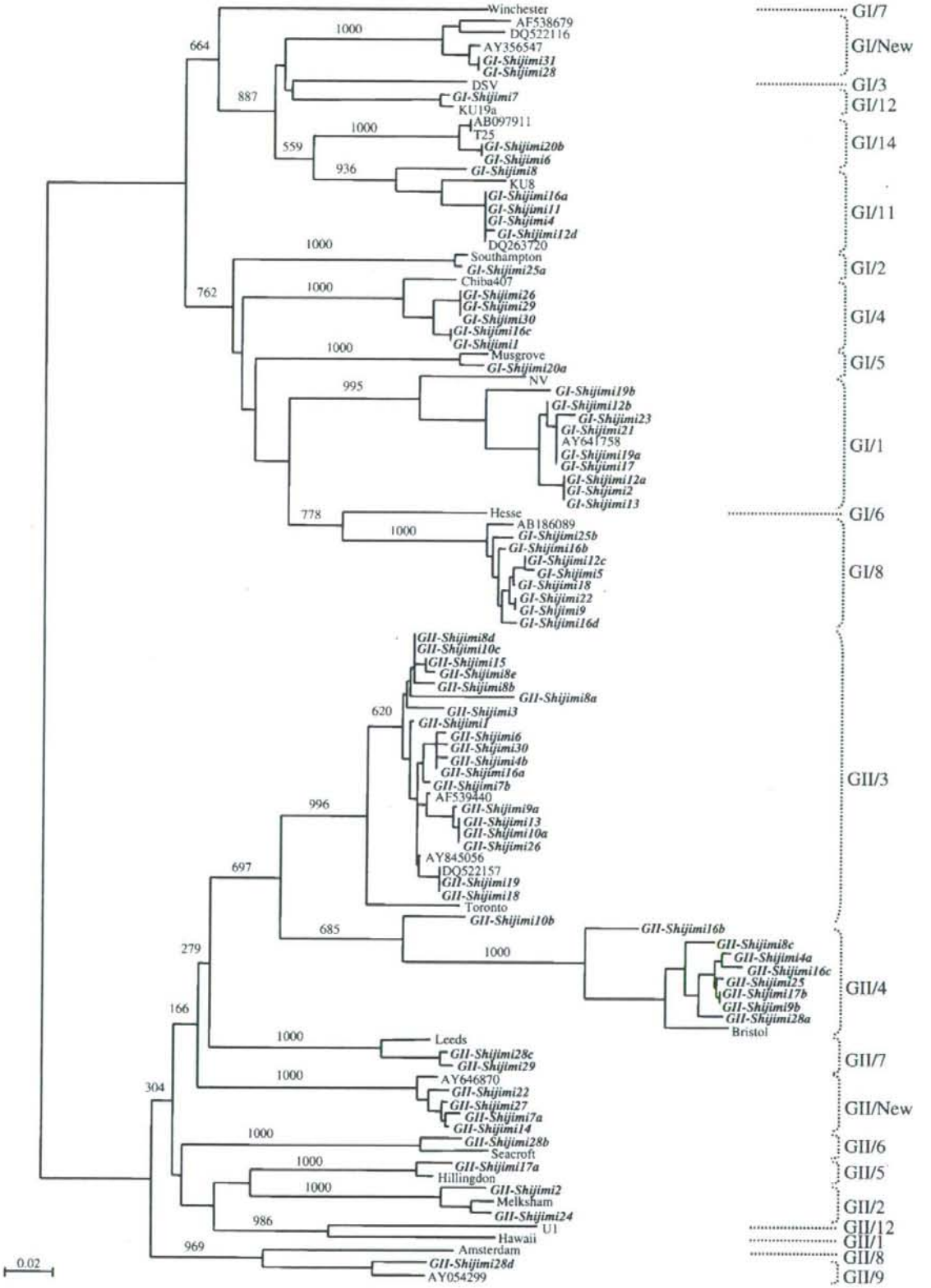
Winchester
AF538679
DQ522116
AY356547
GI-Shijimi31
GI-Shijimi28
DSV
GI-Shijimi7
KU19a
AB097911
T25
GI-Shijimi20b
GI-Shijimi6
GI-Shijimi8
KU8
GI-Shijimi16a
GI-Shijimi11
GI-Shijimi4
GI-Shijimi12d
DQ263720
Southampton
GI-Shijimi25a
Chiba407
GI-Shijimi26
GI-Shijimi29
GI-Shijimi30
GI-Shijimi16c
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Musgrove
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GI-Shijimi13
Hesse
AB186089
GI-Shijimi25b
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GI-Shijimi12c
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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
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Toronto
GII-Shijimi10b
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GII-Shijimi8c
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GII-Shijimi17b
GII-Shijimi9b
GII-Shijimi28a
Bristol
Leeds
GII-Shijimi28c
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AY646870
GII-Shijimi22
GII-Shijimi27
GII-Shijimi7a
GII-Shijimi14
GII-Shijimi28b
Seacroft
GII-Shijimi17a
Hillingdon
GII-Shijimi2
Melksham
GII-Shijimi24
U1
Hawaii
Amsterdam
GII-Shijimi28d
AY054299
664
1000
887
559
936
762
778
620
996
697
685
279
166
304
986
969
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GI/7
GI/New
GI/3
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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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Serological Evidence for Hepatitis E Virus Infection in Laboratory Monkeys and Pigs in Animal Facilities in Japan

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Abstract: *In laboratory animal facilities, monkeys and pigs are used for animal experiments, but the details of hepatitis E virus (HEV) infection in these animals are unknown. The risk of infection from laboratory animals to humans has become a concern; therefore, much attention should be paid to the handling of these animals during their care and use, including surgical procedures performed on infected animals. In this connection, serum samples collected from 916 monkeys and 77 pigs kept in 23 animal facilities belonging to the Japanese Association of Laboratory Animal Facilities of National University Corporations (JALAN) and the Japanese Association of Laboratory Animal Facilities of Public and Private Universities (JALAP) in Japan were examined for the purpose of detecting antibodies to HEV and HEV RNA by using ELISA and RT-PCR, respectively. One hundred and seven serum samples of 916 (11.7%) monkeys were positive for anti-HEV IgG, and 7 and 17 serum samples of 916*

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(0.8% and 5.3%) monkeys were positive for anti-HEV IgM and IgA, respectively. Thirty-six samples from 62 (58.1%) farm pigs were positive for anti-HEV IgG, whereas all samples tested from miniature pigs were negative (0/15, 0%). Seven samples from 62 (9.1%) farm pigs and 7 samples from 916 (0.8%) monkeys were positive for IgM antibody, but these HEV-IgM antibody positive serum samples were HEV-RNA negative by RT-PCR. The IgM antibody positive rate (9.1%) of farm pigs was much higher than that of monkeys (0.8%). These results suggest the relative levels of risk of HEV infection from these animals to animal handlers and researchers who work with them in laboratory animal facilities.
Key words: *anti-HEV antibodies, experimental animals, hepatitis E virus, monkeys, pigs*

Introduction

Hepatitis E virus (HEV) is one of the most important causes of acute hepatitis in many developing countries in Asia [32], the Middle East and North Africa [22], and was recently classified as the sole member of the genus Hepevirus in the family Hepeviridae [5]. Hepatitis E was first recognized when a large water-borne hepatitis outbreak occurred in India in 1955, in which the antibody-positive rate of hepatitis A virus was extremely high in all age groups [9]. Because HEV is transmitted via an oral-fecal route, contaminated drinking water and food are the primary source of this infection. Although hepatitis E is self-limiting and neither chronic nor persistent infection is observed in the adult population in general, a high mortality rate of 15–20% is reported in pregnant women [12].

HEV is a non-enveloped, single-stranded, positive-sense RNA virus [30]. By phylogenetic analysis, at least four major genotypes of HEV have been identified [25]. Genotype 1 (G1) HEV was isolated in Asia and Africa [22, 11], genotype 2 (G2) in Mexico [34], and genotypes 3 (G3) and 4 (G4) in the United States, European countries, China, Japan, and Vietnam [7, 20, 24, 27–29, 35]. These viruses are thought to comprise a single serotype [22].

Although most hepatitis E cases in developed countries were thought to have been imported from developing countries, recent studies have revealed that hepatitis E has occurred in patients who had never been abroad [24, 27]. The findings that genetically similar G3 and G4 HEVs have been isolated from pigs [21, 23], deer and wild boars [15, 20, 31], and that serum antibodies to HEV were detected in a variety of animals including pigs [29], deer, wild boars, wild rats [10], dogs, cats, cows [18, 26], and monkeys [1, 2, 6, 8, 33], suggest that hepatitis E is a zoonosis. Also, hepatitis E virus antibody prevalence among persons who work with swine [4] and among those who live in the rural, southern part of the People's Republic of China [13] has been reported. Recently, direct evidence of G3 HEV transmission from deer and wild boar meats to humans was clearly provided in Japan, suggesting that wild animals are the zoonotic reservoir of HEV in Japan [15, 31]; transmission to humans from pig visceral organs [36], and following surgical training conducted on pigs [3] has also been suspected.

In laboratory animal facilities in Japan, wild Japanese monkeys and pigs from farms are used as experimental animals. Since HEV infection is considered a zoonosis, it is important to pay attention to the possibility of infection in these animals. In this connection, a survey was done to detect HEV infection in monkeys and pigs that were used for animal experimentation. To find HEV infection in laboratory animals, and to examine the HEV infection rate, we performed assays for the detection of anti-HEV antibodies and HEV RNA from monkey sera and pig sera collected from animal facilities belonging to the Japanese Association of Laboratory Animal Facilities of National University Corporations (JALAN) and the Japanese Association of Laboratory Animal Facilities of Public and Private Universities (JALAP) in 2005. Anti-HEV IgG was found in 11.7% of monkeys and 48.6% of pigs that were used for animal experiments, suggesting that animal handlers and researchers should pay attention to HEV infection in laboratory animals. It is considered desirable to use pigs and monkeys that are negative for HEV antibody as often as possible.

Materials and Methods

Monkey and pig sera

Sera (including plasma) were collected from monkeys and pigs that had been used for animal experiments in 1998 and 2005 in 23 animal facilities belonging to JALAN and JALAP. Nine hundred and sixteen samples from monkeys (20 facilities) and 77 samples from pigs (12 facilities) were tested. The pigs included 62 farm pigs and 15 miniature pigs purposely bred for animal experiments. These sera were stored at –30°C until testing.

Preparation of recombinant virus-like particles

A recombinant baculovirus, Ac5480/7126, harboring the G1 HEV capsid protein gene with III amino acids deleted at the N-terminal was constructed as described previously [14]. In brief, Tn5 cells (High Five™, Invitrogen, San Diego, CA) were infected with Ac5480/7126 at a multiplicity of infection of 10 and incubated at 26.5°C for 7 days. The intact cells and cell debris were removed from the culture medium, then the recombinant virus-like particles (VLPs) of 53 kDa were concentrated by centrifugation at 100,000 × g for 2 h in an SW28 rotor (Beckman Instruments, Inc., Fullerton, CA). The VLPs were further purified by isopycnic binding in CsCl gradient [16]. Recombinant baculoviruses that expressed N-terminal truncated capsid proteins of G3 and G4 HEV were similarly prepared, and the VLPs of 53 kDa were also prepared (Li *et al.* unpublished).

Detection of anti-HEV antibodies in monkeys and pigs

Flat-bottom, 96-well, polystyrene microplates (Immulon 2, Dynex Technologies, Inc., Chantilly, VA) were coated with the purified VLPs (1 μg/ml, 100 μl/well). The plates were incubated at 4°C overnight. Unbound VLPs were removed, and the wells were washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and then blocked at 37°C for 1 h with 200 μl of 5% skim milk (Difco Laboratories, Detroit, MI) in PBS-T. After the plates were washed 4 times with PBS-T, monkey serum or pig serum (100 μl/well) was added at a dilution of 1:200 in PBS-T containing 1% skim milk. The plates were incubated at 37°C for 1 h and then washed 4 times as described above. The wells were incubated with 100 μl of peroxidase-conjugated goat anti-human IgG (H+L) (1:5,000 dilution) or anti-swine IgG (H+L) (1:4,000 dilution) (KPL, Guildford, UK) or anti-human IgM (μ;) (1:1,000 dilution) or anti-swine IgM (μ) (1:2,000 dilution) (KPL, Guildford, UK) or anti-monkey IgA (1:1,000 dilution) (Alpha Diagnostic Intl., Inc., San Antonio, TX) in PBS-T containing 1% skim milk. The plates were incubated at 37°C for 1 h and washed 4 times with PBS-T. Next, 100 μl of the substrate orthophenylenediamine (Sigma Chemical Co., St. Louis, MO) and H_2O_2 were added to each well. The plates were incubated in a darkroom at room temperature for 30 min, and then 50 μl of 4 N H_2SO_4 was added to each well. After the plates had stood at room temperature for 10 min, the absorbance at 492 nm was measured.

Western blot assay

Approximately 1 μg of VLPs was separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in 50 mM Tris-HCL (pH 7.4) and 150 mM NaCl, and incubated with monkey serum (1:500 dilution), followed by HRP-goat anti-human IgG (H+L) (1:1,000 dilution). The membrane was treated with ECL detection reagent (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions, and the luminescence was recorded by exposure to film (Fuji Film, Tokyo, Japan).

Detection of HEV RNA by RT-PCR

Total RNA was extracted with RNA zol-LS reagent (Tel-test, Inc., Friendswood, TX) using 200 μl of the monkey serum and then resuspended in 20 μl of DNase-, RNase-, and proteinase-free water. Reverse transcription (RT) was performed at 42°C for 50 min followed by 70°C for 15 min, with 1 μl of the oligo (dT) primer, 1 μl of superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD), 0.5 μl of 0.1 M dithiothreitol, 4 μl of 5 × RT buffer, and 1 μl of 10 mM deoxynucleoside triphosphates. Two microliters of the resulting cDNA were amplified in a 50-μl sample for nested PCR with Ex Taq DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan) with an external sense primer HEV-F1 (5'-TAYCGHAAYCAAGGHTGGCG-3'; nucleotide (nt) residues 5903-5922 of G1 Myanmar strain, GenBank

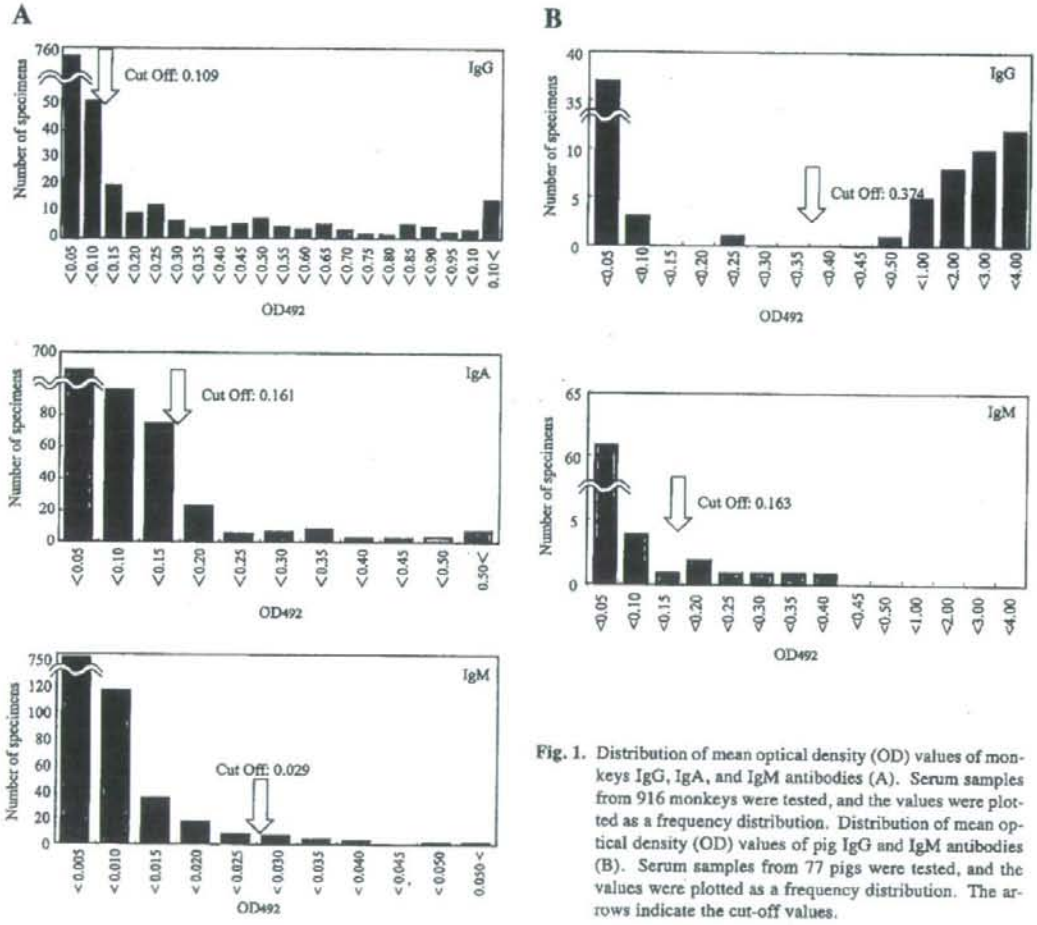

Fig. 1. Distribution of mean optical density (OD) values of monkeys IgG, IgA, and IgM antibodies (A). Serum samples from 916 monkeys were tested, and the values were plotted as a frequency distribution. Distribution of mean optical density (OD) values of pig IgG and IgM antibodies (B). Serum samples from 77 pigs were tested, and the values were plotted as a frequency distribution. The arrows indicate the cut-off values.

D10330) and an antisense primer HEV-R2 (5'-TGYTG-GTTRTCRTARTCCTG-3'; nt residues 6486-6467 of G1 Myanmar strain, GenBank D10330), using the GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA). Each cycle consisted of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and extension reaction at 7°C for 60 sec, followed by final extension at 72 °C for 7 min. The nested PCR was done with the internal sense primer HEV-F2 (5'-GGBGTBGCNGAGGAG-GAGGC-3'; nt residues 5939-5958) and the internal antisense primer HEV-R1 (5'-CGACGAAATYAAT-TCTGTCG-3'; nt residues 6316-6297) under the same conditions [15].

Results

Detection of anti-HEV IgG, IgM, and IgA in monkey sera and anti-HEV IgG and IgM in pig sera

The monkey serum samples were tested to detect anti-HEV IgG, IgM, and IgA at a dilution of 1:200 by ELISA. The distribution of the optical density (OD) values of monkey sera is shown in Fig. 1A. The OD values for IgM antibodies to HEV ranged from 0.000 to 0.136, and no serum samples with a titer of 200 had an OD value higher than 0.20. The OD values of anti-HEV IgG ranged from 0.000 to 1.926, and 83 sera whose titers ranged from 1:200 to 1:51,200 were higher than 0.20.

Table 1. OD values and antibody titers in monkey sera

Sera	OD values (1:200) (IgG/IgM)	Antibody titers (IgG/IgM)
No. 14	0.106/0.01	(<200)/(<200)
No. 15	0.017/0.002	(<200)/(<200)
No. 19	0.113/0.00	200/(<200)
No. 20	0.457/0.00	800/(<200)
No. 21	0.406/0.00	400/(<200)
No. 60	1.157/0.003	3,200/(<200)
No. 59	1.692/0.003	6,400/(<200)
No. 58	1.685/0.018	12,800/(<200)
No. 25	0.529/0.00	800/(<200)
No. 17	0.773/0.002	1,600/(<200)
No. 22	0.707/0.00	1,600/(<200)
No. 137	0.513/0.00	800/(<200)
No. 28	0.903/0.00	1,600/(<200)
No. 46	0.632/0.00	1,600/(<200)
No. 53	1.639/0.003	3,200/(<200)
No. 190	1.926/0.011	51,200/(<200)
No. 323	1.742/0.013	6,400/(<200)
No. 324	1.773/0.008	6,400/(<200)

OD values and antibody titers of IgG/IgM of several monkey samples are summarized in Table 1. The OD values of anti-HEV IgA ranged from 0.000 to 1.096, and 32 sera whose titers ranged from 1:200 to 1:6,400 were higher than 0.20 (data not shown).

The pig serum samples were also tested for the detection of anti-HEV IgG and IgM at a dilution of 1:200 by ELISA. The distribution of the OD values of pig sera is shown in Fig. 1B. The cut-off values, were determined in the same manner as the monkey sera, and are shown in the following section.

Specificity of IgG antibody in monkey sera

To determine whether the IgG antibody detected in monkey sera was specific to HEV, 7 serum samples were selected and examined by western blot assay (Table 1). The G1, G3, and G4 VLPs were separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The serum dilution of 1:500 was used for the assay. As shown in Fig. 2, strong bands with a molecular weight of 53 kDa corresponding to G1, G3, and G4 VLPs were detected in sample Nos. 58, 28, 137, and 22. The OD values of these sera were between 1.844, 0.347, 0.259, and 0.229 as determined by ELISA. Weak bands were detected in sample Nos. 21 and 20, whose OD values were 0.126 and 0.185, respectively, whereas no band was detected in sample No. 15, which had a low OD value of 0.017 as determined by ELISA. These results indicate that the anti-HEV-IgG detected in monkeys by ELISA was specific for HEV. In this survey, farm pigs were considered to be the reservoir of hepatitis E virus. As the IgG antibody detected in pig sera showed very high OD values without samples having OD values as low as those of the negative controls, we considered all positive samples to have antibodies specific to HEV. Therefore, determination by western blot assay was not conducted.

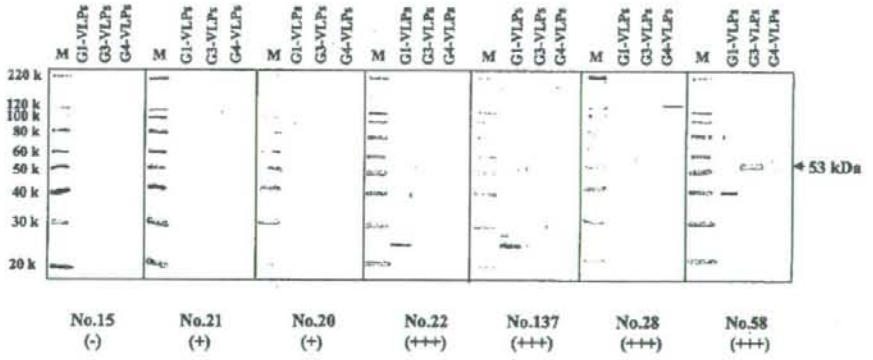

Fig. 2. Specificity of the IgG antibody determined by western blot assay. The genotype 1 (G1), G3, and G4 VLPs were used as the antigens; 7 monkeys' sera with different OD values were evaluated. Strong band (+++), weak band (+), and no band (–) by western blot assay are indicated. M: molecular weight marker.

Table 2. Results of antibody test for hepatitis E virus (HEV) in laboratory animals[a] (positive rates)

			IgG	IgM	IgA
Pigs[b]	Farm pigs	62	36 (58.1%)	4 (6.5%)	NT[c]
	Miniature pigs	15	0 (0%)	0 (0%)	NT
	Total	77	36 (46.8%)	4 (5.2%)	NT
Non-human primates[d]	Cynomolgus monkeys	115	9 (7.8%)	1 (0.9%)	4 (3.5%)
	Rhesus macaque	264	26 (9.8%)	0 (0%)	6 (6.1%)
	Japanese macaque	528	72 (13.6%)	6 (1.1%)	29 (5.5%)
	Taiwan macaque	6	0 (0%)	0 (0%)	0 (0%)
	Pigtail macaque	2	0 (0%)	0 (0%)	0 (0%)
	African green monkeys	1	0 (0%)	0 (0%)	0 (0%)
	Total	916	107 (11.7%)	7 (0.8%)	49 (5.3%)

[a]Samples with the OD values over (the mean of negative samples + 3SD) were classified as positive. [b]Sera (including plasma) of pigs that had been used and kept as laboratory animals. Samples were supplied during the period between August and December in 2005 (77 samples from 12 institutions). [c]Not tested. [d]Sera (including plasma) of non-human primates that had been used and kept as laboratory animals. Samples were supplied during the period between August and December in 2005 (627 samples from 19 institutions) and a part of the samples collected by the Japanese Association of Laboratory Animal Facilities of National University Corporations during the fiscal year of 1998 (289 samples).

Prevalence of anti-HEV IgG, IgM, and IgA in monkey and pig sera

The cut-off values of IgG, IgM, and IgA for the ELISA were determined with 833, 916, and 884 antibody-negative monkey serum samples, respectively. The OD values of anti-HEV IgG of these sera were between 0.000 and 0.193, and the mean value was 0.019 with SD of 0.03. Therefore, the cut-off value, the mean value +3SD, was calculated to be 0.109 (Fig. 1A). When this value was employed, the prevalence of anti-HEV IgG appeared to be 11.7% (107/916). The antibody-positive rate was 13.4% in females and 11.9% in males, however, the difference between the sexes was not statistically significant ($P>0.05$). The mean OD value of anti-HEV IgM for these 916 sera was 0.001 and that of SD was 0.009; thus, the cut-off value was calculated to be 0.029 ($0.001 + 3 \times 0.009$). Using this cut-off value, 7 monkey sera appeared positive for IgM antibody, and therefore the prevalence rate was 0.8% (7/916). The mean OD value of the 884 anti-HEV IgA-negative sera was 0.032 and that of SD was 0.043; thus, the cut-off value was calculated to be 0.161 ($0.032 + 3 \times 0.043$). Using this cut-off value, 17 monkey sera appeared positive for IgA antibody; therefore, the prevalence rate was 5.3% (49/916).

The cut-off value of IgG and IgM for the ELISA was determined with 40 and 70 antibody-negative pig serum samples, respectively. The OD values of anti-HEV IgG of these sera were between –0.662 and 0.248, and the mean value was –0.189 with SD 0.188. Therefore, the cut-off value, the mean value +3SD, was calculated to be 0.374 ($-0.189 + 3 \times 0.188$). When this value was employed, the prevalence of anti-HEV IgG appeared to be 46.8% (36/77). The mean OD value of anti-HEV IgM for these 70 antibody-negative pig sera was –0.049 and that of SD was 0.071; thus, the cut-off value was calculated to be 0.163 ($-0.049 + 3 \times 0.071$). Using this cut-off value, 7 pig sera appeared positive for IgM antibody; therefore, the prevalence rate was 9.1% (7/77).

The summary of the results of the positive rates of HEV antibody in the samples of laboratory animals are shown in Table 2. One hundred and seven serum samples of 916 (11.7%) monkeys were positive for anti-HEV IgG, and 7 and 49 serum samples of 916 (0.8% and 5.3%) monkeys were positive for anti-HEV IgM and IgA, respectively (Table 2).

Thirty-six sera of 77 (46.8%) pigs were positive for anti-HEV IgG. All positive samples were from the 62 farm pigs (36/62, 58.1%), whereas all samples tested from miniature pigs were negative (0/15, 0%). Four samples from farm pigs were positive for IgM antibody. The IgM antibody positive rate (5.2%) of pigs was much higher than that of monkeys (0.8%).

Detection of HEV RNA by RT-PCR

Four serum samples of 77 pigs were positive for HEV-IgM antibody as tested by RT-PCR for HEV RNA, but we were not able to amplify any HEV sequences in these samples.

Discussion

In the Biosafety Committee (Chair: Hiroshi Yamamoto, University of Toyama) of the Japanese Association of Laboratory Animal Facilities of National University Corporations (JALAN), various discussions regarding safety measures for humans have focused on the infection of laboratory animals in laboratory facilities, especially for zoonosis. As a part of this activity, a survey for B virus antibody in non-human primates was done and the results were published (*Exp. Anim.* 1998, 47: 199–202). In recent years, social concerns have been focused on hepatitis E transmitted from animals to humans. A survey on the prevalence of hepatitis E was conducted in laboratory animals kept in animal facilities belonging to the JALAN and the JALAP. Hepatitis E virus (HEV) is said to be a zoonosis and classified as Category IV by the Law Concerning Prevention of Infection of Infectious Diseases and Patients with Infectious Diseases in Japan.

In recent years, more pigs have been used as laboratory animals than in the past. Farm pigs, in which a high level of hepatitis E infection was reported, are used for animal experiments in medical and pharmaceutical research. Therefore, occupational health and safety measures for animal handlers and researchers are urgently needed. Non-human primates are also extremely important as laboratory animals for medical research. Chimpanzees, tamarins, African green monkeys, rhesus macaques and crab-eating macaques appear to be sensitive to HEV. Therefore, HEV infection was surveyed in monkeys and pigs kept in laboratory animal facilities. Based on the survey results, it is clear that persons working with laboratory animals should be well informed about HEV infection in laboratory animal facilities and safety measures to prevent this disease. For that purpose, the survey results are outlined and reported here.

An ELISA with recombinant HEV VLPs was used to detect anti-HEV IgG, IgM, and IgA antibodies in non-human primates and anti-HEV IgG and IgM in pigs. This assay was capable of detecting anti-HEV antibodies in human sera with high sensitivity and specificity [17]. The HEV specificity of the ELISA in monkey sera was confirmed by western blot assay in this survey. Through examination of pigs and monkeys kept in laboratory animal facilities, anti-HEV IgG-specific monkey antibody was detected. HEV antibody-positive rates were 60, 0, 13, 10, and 8 percent for farm pigs, miniature pigs, Japanese macaques, rhesus macaques, and crab-eating macaques, respectively. In general, farm pigs are thought to be highly contaminated with HEV. As the survey results indicate, researchers must pay due attention when using farm pigs for experiments. Generally, the possibility that antibody-positive animals, especially animals positive for IgG and IgA antibodies, carry or excrete HEV is fairly low. On the other hand, 7 farm pigs were confirmed to be positive for HEV IgM antibody. These animals may have been carrying HEV. Therefore, we thought it was necessary to conduct testing with PCR. However, we were not able to amplify any HEV sequences in these samples. Takahashi *et al.* suggested that pigs of 3 or 4 months age that were introduced from HEV positive farms sometimes carry the virus and excrete it in their feces [29]. Therefore, when using farm pigs for animal experiments, we should introduce 1–4 months pigs which are HEV negative to laboratory animal facilities. As for monkeys, our testing revealed that 107 serum samples of 916 animals tested were positive for anti-HEV IgG antibody, but there were no animals strongly positive for anti-HEV IgM antibody. There may have been no animals showing the symptom of viremia at the time of blood sampling. Therefore, the monkeys tested in this survey are considered not to be hazardous for use in experiments. It is notable that no infection with HEV was observed in miniature pigs purposely bred for experiments, though they were few in number in our sample. The safety of laboratory miniature pigs was confirmed with respect to HEV contamination.

Our study results confirm that past reports of pigs and monkeys being positive for HEV antibody in Japan [8, 29] were correct; however, no report of HEV infection attributable to pigs and monkeys that were used in experiments has been found so far. Three cases of HEV in humans were reported in the current Infectious Diseases

Weekly Report Digest (dated 14 April 2006), and one of them was attributable to consumption of pig liver. Uncooked meat of laboratory animals is never consumed by humans in Japan. The possibility remains, however, that HEV infection may occur due to accidental injection and injuries during experimental procedures and oral infection during animal handling. Furthermore, HEV antibody prevalence among persons who work with swine [4] and among those who live in the rural southern of the People's Republic of China [13] has been reported. Renou *et al.* and Colson *et al.* reported the possible zoonotic transmission of HEV through direct contact between a pet pig and its owner, and from surgical training on pigs, respectively, in France in 2007 [3, 23]. In swine, the duration of viremia after HEV infection is not very long [19]. IgM and IgG antibodies rapidly increase in the blood after infection, and infection risk decreases due to the increased antibody levels. In experiments using adult pigs, there is little possibility of encountering viremic animals; however, the infection pattern among animals is not clear, and the possibility of infection due to failures of experimental procedures cannot be denied. Therefore, animals from HEV-negative colonies should be used. By avoiding direct contact with blood and organs as well as paying close attention during washing procedures that may involve the abundant spraying of feces, it is possible to prevent infection from laboratory animals of humans.

It is very rare for animal handlers to become infected with HEV while taking care of animals positive for HEV antibody if they wear proper protective equipment against infection, such as masks *et cetera*, according to ordinary husbandry procedures. In large-scale laboratory animal rooms, animal handlers should use appropriate protective measures, because splashing occurs during some procedures, such as washing. When disposing of animals at the end of an experiment, if it has been confirmed that those animals were in the viremic state, workers must pay attention to the possibility of infection via blood and increase their safety precautions. Especially among pregnant women, HEV has a high infection rate and there is a high onset rate of fulminant hepatitis [12]. Khuroo *et al.* reported that the fulminant rate in pregnant women (22.2%) was higher than that in non-pregnant women (0%) and men (2.8%), and all cases of fulminant hepatitis in pregnancy occurred in patients who were in the last trimester [12]. In Japan the Labour Standards Law guarantees that a woman who is pregnant can take holidays for 6 weeks before birth and 8 weeks after childbirth, respectively, but holiday periods are not enough for preventing HEV infection. Therefore, animal handlers and researchers working with pigs and monkeys who are pregnant should be able to change their duties to other duties in the animal facilities during the last trimester. It is necessary for people at special risk to be exceptionally careful when they engage in work or experiments presenting possible exposure to HEV infection.

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