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SHORT REPORT: DETECTION OF HEPATITIS E VIRUS RNA FROM THE BIVALVE YAMATO-SHIJIMI (*CORBICULA JAPONICA*) IN JAPAN

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Abstract. To evaluate whether bivalves are contaminated with hepatitis E virus (HEV), samples of a bivalve called Yamato-Shijimi (*Corbicula japonica*) were examined for HEV by reverse transcription–polymerase chain reaction. Genotype 3 HEV was detected from 2 of 32 packages of Yamato-Shijimi obtained from Japanese rivers, which indicated that HEV contaminates river water in Japan.

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus recently classified as the sole member of the genus *Hepevirus* in the family Hepeviridae.[1,2] This virus causes human hepatitis E and is transmitted primarily by the fecal-oral route through contaminated drinking water.[3,4] However, recent studies have demonstrated that various animal species have serum antibodies to HEV, and its viral genome has been detected in swine, wild deer, wild boar, and mongoose, which suggests that hepatitis E is a zoonotic disease.[5–10] Because HEV is excreted into feces,[11,12] there is a risk of HEV contamination in environmental water. In fact, HEV has been detected in sewage from industrialized countries, including Spain, the United States, and France.[13–15] However, HEV contamination of river water has not been examined. In the present study we detected the HEV genome from a bivalve called Yamato-Shijimi (*Corbicula japonica*), which suggested that river water in Japan is contaminated with HEV.

A total of 32 packages of Yamato-Shijimi were obtained at a fish market in December 2005 and March 2006. All samples were harvested at areas A, B, C, D, E, F, G, and H in western Japan. The package numbers and collection days are shown in Table 1. The samples were shucked, and the digestive diverticulum were removed by dissection and weighed. One gram of digestive diverticulum obtained from 10–15 Yamato-Shijimi was homogenized with an Omni-mixer (OCI Instruments, Waterbury, CT) in 10 mL of phosphate-buffered saline, pH 7.4, for two 30-second intervals at a maximum speed of 18,000 rpm. After centrifugation at 10,000 × *g* for 30 minutes at 4°C, the supernatant was centrifuged at 100,000 × *g* for 2 hours in an SW41 rotor (Beckman Instruments, Inc., Fullerton, CA). The pellet was resuspended in 140 μL of distilled water and stored at -80°C until use.

Total RNA was extracted with the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) and resuspended in 20 μL of DNase-, RNase-, and proteinase-free water. Reverse transcription (RT) was performed at 42°C for 50 minutes, followed by 70°C for 15 minutes in a 20-μL reaction mixture containing 1 μL of Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA), 1 μL of oligo (dT) primer, 1 μL of RNaseOUT™ (Invitrogen), 2 μL of 0.1 M dithiothreitol, 4 μL of 5× RT buffer, 1 μL of 10 mM deoxynucleoside triphosphates, 5 μL of RNA, and 5 μL of distilled water. An RT–polymerase chain reaction was performed to amplify part of the open reading frame 2 (ORF2) as described previously.[8,10] Two microliters of the cDNA was used for the first PCR in a 50-μL reaction mixture with external forward primer HEV-F1 (5′-GGBGTBGCNGAGGAGGAGGC-3′) and external reverse primer HEV-R2 (5′-TGYTGGTTRT-CRTARTCCTG-3′), which corresponded to nucleotide residues 5903–5922 and 6486–6467, respectively, of the G1 Myanmar strain (D10330). Each cycle consisted of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by final extension at 72°C for 7 minutes. Two microliters of the first PCR product was used for a nested PCR with internal forward primer HEV-F2 (5′-TAYCGHAAYCAAGGHTG-GCG-3′; nucleotide residues 5939–5958) and internal reverse primer HEV-R1 (5′-CGACGAAATYAATTCTGTCG-3′, nucleotide residues 6316–6297) under the same conditions.

Two packages, B4 and B6, collected in area B on February 7, 2006, and March 1, 2006, were positive for HEV RNA by

TABLE 1
Detection of hepatitis E virus (HEV) in *Corbicula japonica*

Package no.	Collection day	HEV RNA
A1	12/08/05	–
A2	12/22/05	–
A3	1/14/06	–
A4	1/22/06	–
A5	1/22/06	–
A6	2/05/06	–
A7	2/17/06	–
A8	3/02/06	–
A9	3/14/06	–
A10	3/15/06	–
B1	12/10/05	–
B2	12/17/05	–
B3	1/24/06	–
B4	2/07/06	+
B5	2/19/06	–
B6	3/01/06	+
B7	3/17/06	–
B8	3/18/06	–
C1	1/10/06	–
D1	1/16/06	–
D2	1/20/06	–
D3	3/14/06	–
E1	1/21/06	–
E2	1/26/06	–
E3	2/25/06	–
E4	3/10/06	–
F1	3/13/06	–
F2	3/14/06	–
F3	3/18/06	–
F4	3/18/06	–
G	3/18/06	–
H	3/18/06	–

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RT-PCR. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and cloned into TA cloning vector pCR2.1 (Invitrogen). The nucleotide sequence of each of 10 clones was determined. Most of the sequences formed a single genotype 3 cluster. The exceptions (B4-13) formed a different cluster along with Sakai-9 detected from a wild boar in 2004 in Japan (Figure 1). We found large numbers of small different nucleotide sequences among the clones with 88.9–100% identity, even when they were derived from the same package, which indicated that multiple HEV strains were accumulated in the digestive diverticulum of Yamato-Shijimi.

To further analyze the HEV RNA detected in the Yamato-Shijimi, the entire ORF2 of B4 RNA was amplified as overlapping segments, and the nucleotide sequences were determined. The full-length ORF2 consisted of 1,980 basepairs and were phylogenetically classified into genotype 3. High amino acid identities (97.57–98.87%) were observed with HEV strains detected from hepatitis E patients, swine, wild boar, and wild deer in Japan. This is the first report on the detection of HEV from a bivalve.

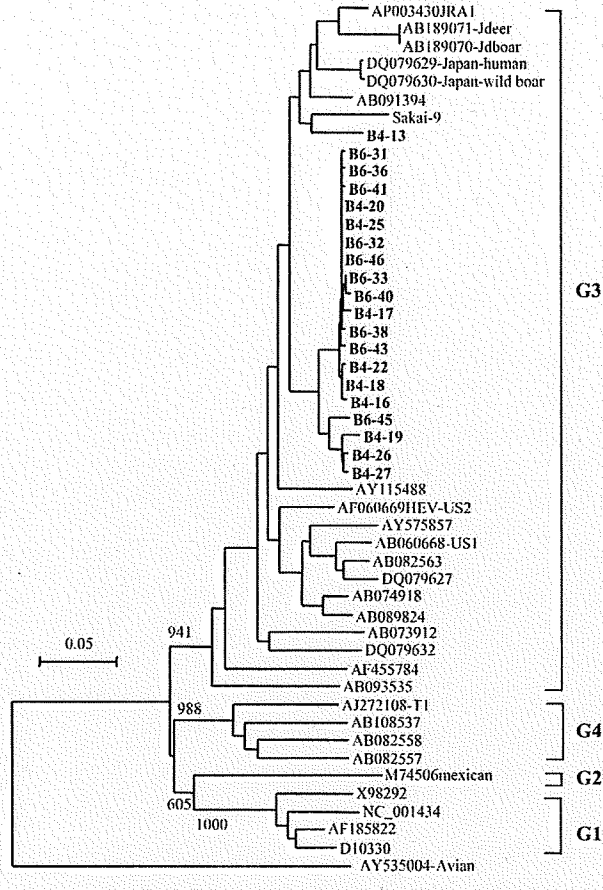

FIGURE 1. Phylogenetic trees of hepatitis E virus (HEV) constructed with avian HEV as an outgroup. A partial open reading frame 2 ORF2 (338 basepairs) of each of 10 clones of B4 (B4-13, B4-16, B4-17, B4-18, B4-19, B4-20, B4-22, B4-25, B4-26, and B4-27) and B6 (B6-31, B6-32, B6-33, B6-36, B6-38, B6-40, B6-41, B6-43, B6-45, and B6-46) were analyzed by the neighbor-joining method. The bootstrap values correspond to 1,000 replications. All nucleotide sequences determined in this study are shown in bold. Other HEV sequences were obtained from GenBank.

The HEV sequences were detected from Yamato-Shijimi (*Corbicula japonica*) harvested on February 7, 2006, and March 1, 2006, in western Japan. The Yamato-Shijimi, a brackish-water bivalve, grows in sandy mud in or near rivers, ponds, and lakes. During breathing and feeding, this bivalve filters a large amount of water. When the water is contaminated with HEV, the virus is ultimately concentrated in the digestive diverticula of the bivalves.

The source of HEV in this organism is not known. Since Japan was considered not to be endemic for this virus, and disposal of sewage is in this country is efficient, the risk of HEV contamination from human stool was believed to be low. There have been no outbreaks of hepatitis E in Japan from drinking water. However, HEV has been detected in wild deer, wild boar, and mongoose in Japan, and HEV shed in the feces of these animals may pollute environmental water. Wild deer and wild boar are controlled in Japan to eliminate their ability to damage agriculture and forestry; hunting is the main control strategy. Hunters usually wash killed animals in river water and this would increase the risk of HEV contamination in river water. These wild animals presumably play an important role in the contamination of environmental water.

In Japan, many outbreaks caused by bivalves contaminated with hepatitis A virus and noroviruses have been reported. Fortunately, Yamato-Shijimi is generally eaten as an ingredient in hot miso soup in Japan, and the heat, usually at 100°C for nearly 10 minutes, decreases the risk of HEV transmission from Yamato-Shijimi to humans. However, more efforts are needed to determine the infectivity and stability of HEV in the natural environment, including that in Yamato-Shijimi.

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Brief Report

Prevalence of antibody to hepatitis E virus among wild sika deer, *Cervus nippon*, in Japan

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Summary

We examined 976 sika deer serum samples, 159 liver tissue samples and 88 stool samples collected from 16 prefectures in Japan, and performed ELISA and RT-PCR assays to detect antibodies to HEV and HEV RNA, respectively. Although 25 (2.6%) of 976 samples were positive for anti-HEV IgG, the antibody titers were very low. The OD values ranged between 0.018 and 0.486, forming a single distribution rather than a bimodal distribution, suggesting that the antibody detected in this study was not induced by HEV infection, or that deer have low sensitivity to HEV. HEV RNA was not detected in these samples, also suggesting that deer may not play a role as an HEV reservoir.

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Hepatitis E virus (HEV), the sole member of the genus *Hepevirus*, is the causative agent of type E

acute hepatitis in humans [3] . HEV does not have an envelope and is likely to have icosahedral symmetry. The genome is a positive-sense single-stranded polyadenylated RNA molecule, and the 5′ end is capped [11] . The genome of HEV contains three open reading frames, ORF1, ORF2, and ORF3. ORF1 encodes 1693 amino acids (aa) encompassing nonstructural proteins involved in viral replication. ORF2 encodes a 660-aa capsid protein. ORF3 encodes a 123- or 114-aa protein of unknown function [23, 28].

To date, at least four major genotypes of HEV have been identified by phylogenetic analyses. Genotype 1 (G1) HEV was isolated from Asia and Africa [16, 18], genotype 2 (G2) from Mexico [26], Namibia and Nigeria [2, 12], and genotypes 3 (G3) and 4 (G4) from the United States, European countries, China, Taiwan, Japan and Vietnam [4, 13, 17, 19, 27–29]. These viruses are thought to comprise a single serotype [16].

Transmission of human HEV occurs primarily by the fecal-oral route through contaminated water in developing countries [1, 5]. Since 1997, when the first animal strain of HEV was isolated from swine in the United States, there has been much indirect and direct evidence indicating that hepatitis E is a zoonosis and that humans appear to be at risk of infection with swine HEV by cross-species infection [13–15]. Recently, direct evidence of HEV transmission from wild boar (*Sus scrofa*) to humans was provided in Japan, suggesting that these animals are the main zoonotic reservoir of HEV in this country [9]. Indirect evidence of HEV transmission from swine to humans has also been accumulated [22, 30].

Because a case of HEV infection from sika deer meat was reported by Tei et al., sika deer have been considered a possible reservoir in Japan [24, 25]. However, there is only limited surveillance data of HEV infection in deer. In this study, we collected serum samples from wild deer and examined them for the presence of anti-HEV IgG by an antibody ELISA using recombinant virus-like particles (VLPs) as the antigen. We also attempted to detect HEV RNA in serum, stool, and liver samples from the wild deer by RT-PCR analysis.

Between 2003 and 2006, 866 serum samples were collected from wild deer captured in Hokkaido, Iwate, Tochigi, Chiba, Nagano, Aichi, Mie, Hyogo, Shimane, Hiroshima, Oita, Fukuoka, Kumamoto, Miyazaki, and Kagoshima prefectures, and 110 serum samples were collected in 1991–1993 from a deer farm, where the deer were introduced from the habitat at Miyagi prefecture (Fig. 1). In Hyogo Prefecture, an estimated age of 0–10 years was assigned by the tooth replacements and counting cementum annuli of the fist incisors [6]. A total of 88 stool samples were collected from deer captured in Hokkaido, Iwate, Tochigi, Chiba, Nagano, Mie, Hyogo, Hiroshima, Oita, Fukuoka, Kumamoto, Miyazaki, and Kagoshima from 2004 to 2006. They were resuspended in 10 mM phosphate-buffered saline (PBS) to prepare a 10% suspension, shaken at 4 °C for 1 h, and clarified by centrifugation at $10{,}000 \times g$ for 20 min. A total of 159 deer liver tissue were collected from Hyogo (50), Iwate (11) and Hokkaido (98) from 2003 to 2006. The tissue was resuspended in lysis buffer (Qiagen, Inc.) and homogenized. All of the specimens were stored at −20 °C until use.

Serum anti-HEV IgG antibody was detected by ELISA by the method described previously with slight modification [8]. Briefly, a flat-bottom 96-well polystyrene microplate (Immulon 2; Dynex Technologies, Inc. Chantilly, VA) was coated with the purified VLPs (1 μg/ml, 100 μl/well) derived from the G1 Myanmar strain [7]. 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, deer serum (100 μl/well) was added in duplicate 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 rabbit anti-deer IgG (H + L) (1:1000 dilution) (KPL, Guildford, UK) in PBS-T containing 1% skim milk. The plates were incubated at 37 °C for 1 h and washed 4 times with PBS-T. Then, 100 μl of the substrate orthophenylenediamine (Sigma Chemical Co., St. Louis, MO) and H_2O_2 was added to each well. The plates were incubated in a dark room at room temperature for 30 min, then

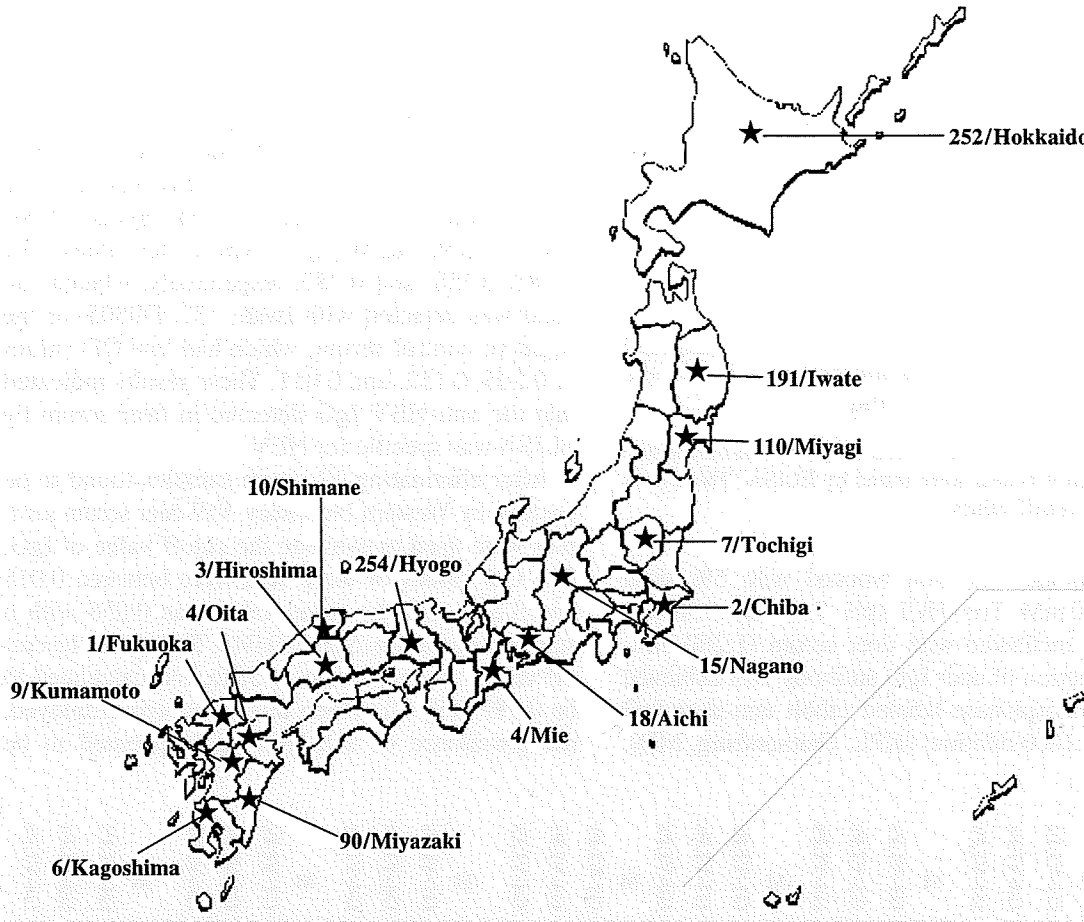

Fig. 1. Numbers and prefecture of captured wild sika deer

50 µl of 4N 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.

Anti-HEV IgG-positive serum was obtained from experimentally immunized captive sika deer that had been shown to be negative for HEV IgG by ELISA. The first and second immunizations were performed with purified VLPs (100 µg) in Freund's complete adjuvant by intramuscular injection at intervals of 2 week. After 2 weeks, the deer received booster injections of the same amount of VLPs in Freund's incomplete adjuvant. The deer was bled one week after the last booster injection. Pre-immunization serum was collected before administration and used as the negative control. Anti-HEV IgG-positive serum and pre-inoculation serum were stored at −30 °C. The anti-HEV IgG titer of the positive serum was 1:3,276,800.

Deer serum samples were tested for anti-HEV IgG at a dilution of 1:200 by ELISA. The distribution of the optical density (OD) values is shown in Fig. 2. The OD values of anti-HEV IgG ranged from 0.018 to 0.486 with the highest antibody titers being 1:400, and formed a single distribution. To determine whether the IgG antibody detected in deer sera was specific for HEV, the positive control serum and negative control serum, and the sera whose OD values were higher than 0.150 were selected and examined by Western blot assay. Approximately 1 µg of the VLPs derived from G1, G3, and G4 HEV was separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane.

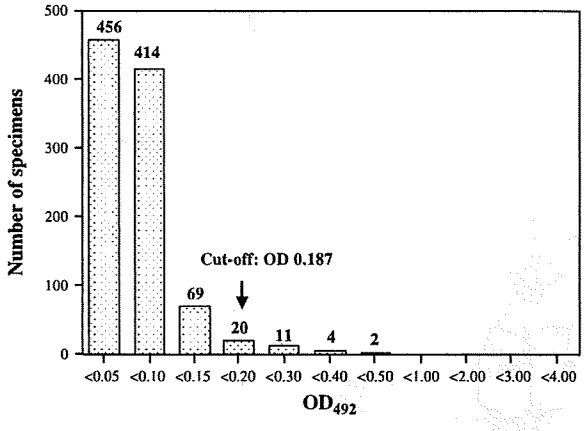

Fig. 2. Distribution of OD values of IgG antibodies. Serum samples from 976 deer were tested by ELISA. The arrows indicate the cutoff values

The membrane was then blocked with 5% skim milk in 50 mM Tris–HCl (pH 7.4) and 150 mM NaCl, and incubated with deer serum (1:200 dilution). Detection of deer IgG antibody was achieved by using phosphatase-labeled rabbit anti-deer IgG (H + L) (1:1000 dilution) (KPL, Gaithersburg, MD). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate P-toluidine were used as coloring agents (Bio-Rad Laboratories, Hercules, CA). As shown in Fig. 3, strong bands with a molecular weight of 53 k corresponding to the G1, G3 and G4 VLPs were detected with positive control sera. Weak bands were detected with Hyogo 0588, Hyogo 0409, and Miyagi 1, whose OD values were 0.486, 0.358, and 0.287, respectively, whereas no band was detected with Iwate 137, D0505, or the negative control serum, which had low OD values of 0.205, 0.152, and 0.051. These results indicated that the anti-HEV IgG detected in deer serum by ELISA was specific for HEV.

After eliminating 17 serum samples found to be positive by Western blot assay, 959 deer serum samples were used to evaluate the cutoff value of IgG. The OD values of these sera were between 0.018 and 0.248, and the mean value was 0.058 with a standard deviation (SD) of 0.043. Therefore, the cutoff value, the mean value + 3SD, was calculated to be 0.187 (Fig. 2). When this value was employed, the prevalence of anti-HEV IgG appeared to be

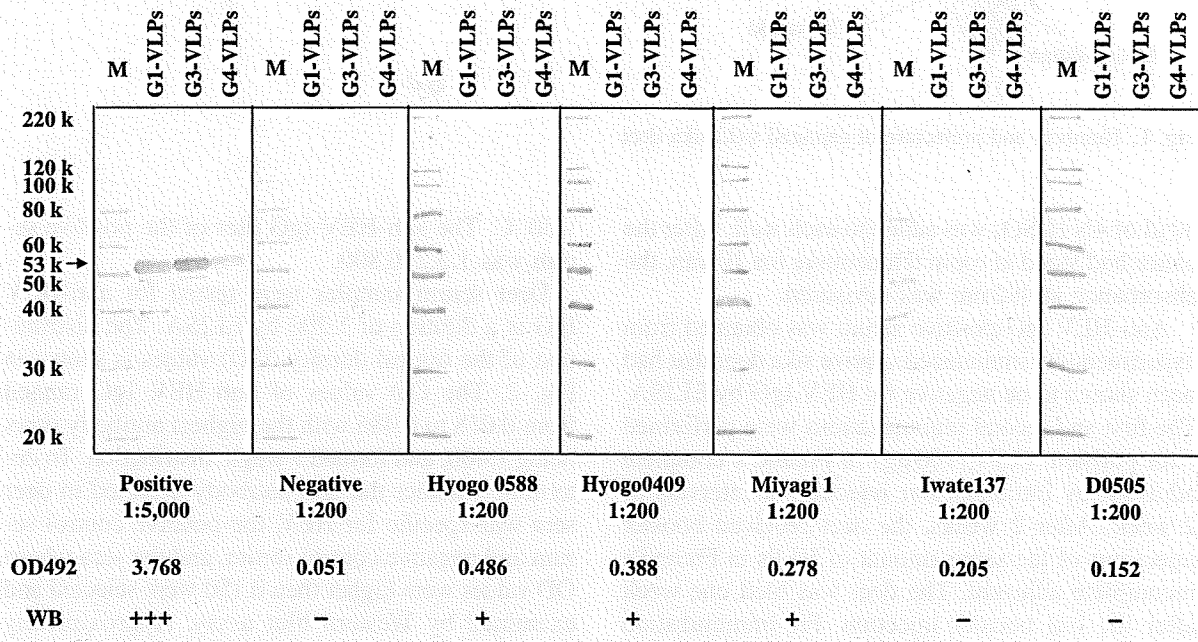

Fig. 3. Specificity of the IgG antibody determined by Western blot assay. The G1, G3, and G4 VLPs were used as the antigens, and 7 deer sera with different OD values were evaluated. The results of the Western blot assay are indicated as +++ (strong band), + (weak band), or – (no band). *M* Molecular weight marker

2.6% (25/976). The antibody-positive rate was 1.2% in Hokkaido, 2.2% in Miyazaki, 3.1% in Iwate, 3.1% in Hyogo, and 3.6% in Miyagi. The difference among these prevalence was not statistically significant ($P > 0.05$).

Eighty-eight paired stool and serum samples from deer captured in Hokkaido (10), Iwate (23), Tochigi (7), Chiba (2), Nagano (5), Mie (4), Hyogo (8), Hiroshima (3), Oita (4), Fukuoka (1), Kumamoto (9), Miyazaki (6), and Kagoshima (6), 166 deer serum samples obtained in Aichi (18), Hyogo (28), Nagano (10), and Miyagi (110), and 159 deer liver tissue samples collected in Hyogo (50), Iwate (11) and Hokkaido (98) were tested by RT-PCR for HEV RNA. Total RNA was extracted with RNAzol-LS reagent (Invitrogen, Inc., Carlsbad, CA) using 200 µl of the deer serum, and 10% stool suspension Reverse transcription (RT) was performed at 42 °C for 50 min followed by 70 °C for 15 min in 20 µl reaction mixture containing 1 µl of Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Inc., Carlsbad, CA), 1 µl of the oligo (dT) primer, 1 µl of RNaseOUT™, 2 µl of 0.1 M dithiothreitol, 4 µl of 5 × RT buffer, 1 µl of 10 mM deoxynucleoside triphosphates, 5 µl of RNA, and 5 µl of distilled water. Two microliters of the resulting cDNA was amplified in a 50 µl reaction mixture containing ExTaq DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan) with an external sense primer HEV-F1 (5′-GGBGTBGCNGAGGAGGAGGC-3′, nucleotide (nt) residues 5903–5922 of G1 Myanmar strain, D10330) and an external antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-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 an extension reaction at 72 °C for 60 sec followed by a final extension at 72 °C for 7 min. The nested PCR was done by using 2 µl of the first PCR product with an internal sense primer HEV-F2 (5′-TAYCGHAA YCAAGGHTGGCG-3′; nt residues 5939–5958) and an internal antisense primer HEV-R1 (5′-CGACGAAATYAATTCTG TCG-3′, nt residues 6316–6297) under the same conditions [9, 10]. Total RNA in deer liver was extracted from 100 mg of the tissue using an RNeasy Mini Kit (Qiagen, Inc.) and dissolved in 50 µl nuclease-free distilled water. The nested RT-PCR was carried out as described by Takahashi et al. [20]. However, we were not able to amplify any HEV sequences in these samples.

An ELISA with recombinant VLPs was used to detect anti-HEV IgG in sika deer in Japan. This assay was previously shown to be capable of detecting anti-HEV antibodies in human and mongoose sera with high sensitivity and specificity [8, 10]. To establish a system for detecting anti-HEV IgG in deer, we first prepared a positive control serum by immunizing deer with the G1 VLPs. After two doses of immunization, an antibody whose titer was as high as 1:3,276,800 was obtained. The specificity of this antibody was confirmed by Western blot assay, indicating that G1 VLPs was an excellent antigen to induce a strong immune response in deer.

In the present study, we tested a total of 976 deer serum samples for the presence of anti-HEV IgG antibody and made the following observations. First, the antibody prevalence was low in sika deer in Japan. Only 25 of 976 (2.6%) samples were positive for anti-HEV IgG by ELISA, which is lower than the prevalence in pigs (58%) and wild boars (44%), both of which are thought to be reservoirs of HEV in Japan [10, 21]. Second, the OD value and titer of anti-HEV IgG were low in deer. The highest OD value was 0.486 and the highest titer was 1:400. This observation is also different from that in pigs and wild boars, where the highest OD values were greater than 3.000 and the titers were greater than 1:51200. Third, the distribution of OD values indicated that only one peak was less than 0.486. The bimodal distribution observed in pigs and wild boars was not seen in deer, indicating that the rate of infection by HEV under natural conditions is extremely low in deer, and suggesting that deer do not play an important role as a reservoir of HEV in Japan.

This study included 254 serum samples from deer captured in Hyogo, where the first deer positive for HEV RNA was found [24]. The prevalence of the antibody-positive rate was 3.1% (5/132) in female and 2.5% (3/122) in male deer, and the difference

between the sexes was not statistically significant ($P > 0.05$). These antibody-positive rates are also not significantly different from those in other areas, including Hokkaido, Iwate, and Miyazaki prefecture. The age of anti-HEV IgG-positive deer was 0–8 years, and no significant correlation between age and prevalence was observed. We also tested HEV RNA in 36 serum samples from deer captured in the same area in Hyogo prefecture where the deer that was positive for HEV RNA was reported. However, we were not able to amplify any HEV sequences in these samples.

Since wild boars are prevalent throughout Japan, with the exception of Hokkaido, and they seem to be eventually infected with HEV, the virus is spread throughout their habitat via their stools. Because wild deer and wild boars share this environment, wild deer might be exposed to HEV. Only low-titer anti-HEV IgG was detected in deer serum in this study, suggesting that either the antibody detected in this study was not induced by HEV infection or that deer have low sensitivity to HEV. If deer were to occasionally come into contact with a small amount of HEV, but were not susceptible to HEV, then a strong immune response to HEV might not be induced.

In summary, the prevalence of anti-HEV IgG in sika deer was lower than the prevalence in two possible reservoirs, pigs and wild boars, and no HEV RNA was detected in 254 sera, 88 stool and 159 liver tissue samples, indicating that wild deer may not be a reservoir of HEV in Japan.

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Crystallization and preliminary X-ray diffraction analysis of recombinant hepatitis E virus-like particle

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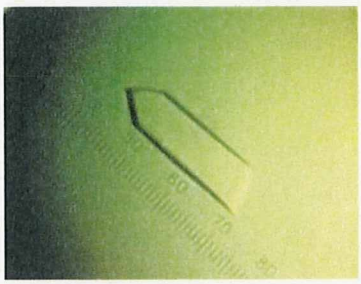


Hepatitis E virus (HEV) accounts for the majority of enterically transmitted hepatitis infections worldwide. Currently, there is no specific treatment for or vaccine against HEV. The major structural protein is derived from open reading frame (ORF) 2 of the viral genome. A potential oral vaccine is provided by the virus-like particles formed by a protein construct of partial ORF3 protein (residue 70–123) fused to the N-terminus of the ORF2 protein (residues 112–608). Single crystals obtained by the hanging-drop vapour-diffusion method at 293 K diffract X-rays to 8.3 Å resolution. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters $a = 337$, $b = 343$, $c = 346$ Å, $\alpha = \beta = \gamma = 90°$, and contain one particle per asymmetric unit.

1. Introduction

The hepatitis E virus (HEV) is a naked icosahedral capsid with a single-stranded positive-sense RNA of 7.2 kbp. The genome encodes three open reading frames (ORFs). ORF1, mapped to the 5′-terminus, encodes nonstructural proteins that are mainly involved in virus replication and protein processing. ORF2, mapped to the 3′-terminus, encodes a viral capsid protein of 660 amino acids that has been found to elicit neutralizing antibodies (Meng *et al.*, 2001; Schofield *et al.*, 2000). ORF3, mapped between ORF1 and ORF2, encodes a protein of 123 amino-acid residues that may interfere with control functions within the infected cell, as summarized by Panda *et al.* (2007). When the N-terminally truncated ORF2 protein (residues 112–660) was expressed with a recombinant baculovirus in an insect-cell line, self-assembling virus-like particles (VLPs) were released into the cell supernatant (Li *et al.*, 1997). These VLPs have been shown to induce anti-HEV antibodies when orally administered to experimental animals (Li *et al.*, 2004). By N- and C-terminal sequencing, the VLP-forming protein was found to be composed of residues 112–608 of the ORF2 protein (VLP_{ORF2}); thus, 52 residues at the C-terminus were cleaved during VLP formation (Li *et al.*, 1997). We have previously reported the structure of HEV-VLP_{ORF2} obtained using electron cryomicroscopy (cryo-EM), which provided a preliminary understanding of the quaternary arrangement of the viral capsids. A three-dimensional reconstruction of VLP_{ORF2} displays $T = 1$ icosahedral symmetry and is composed of 60 copies of the truncated ORF2 protein (Xing *et al.*, 1999; Li *et al.*, 2005).

Although a truncated ORF2 polypeptide is undergoing clinical trials as a vaccine candidate (Shrestha *et al.*, 2007), to date no specific treatment or vaccine has been licensed for HEV (Purcell & Emerson, 2008). The viral capsid is an important form of presenting the conformation-dependent epitopes (Maloney *et al.*, 2005) and HEV-VLP_{ORF2} has been proposed as a suitable candidate for an oral vaccine (Li *et al.*, 2004). Further investigations of the high-resolution structural features of a VLP are required in order to establish the folding and interactions of the viral protein in the context of the HEV particle form, as well as to characterize the immunogenic epitopes that are responsible for inducing the neutralizing antibodies. In the present study, we describe the crystallization and preliminary crys-

tallographic characterization of a purified HEV-$VLP_{ORF3/ORF2}$ capsid composed of a fusion protein obtained by inserting a fragment of ORF3 (residues 70–123) at the N-terminus of the ORF2 peptide including residues 112–608.

2. Experimental procedures and results

2.1. Expression and purification of recombinant HEV-VLPs

Recombinant HEV-$VLP_{ORF3/ORF2}$ was produced using a similar approach to those described previously (Li *et al.*, 1997; Xing *et al.*, 1999) except that an ORF3/ORF2 fusion protein containing a fragment of the ORF3 protein (residues 70–123) attached without an intervening sequence to the N-terminus of a truncated ORF2 protein (residues 112–608) was used in the construct for expression. The transfer vector was co-transfected with insect Sf9 cells (Riken Cell Bank, Tsukuba, Japan) to produce the recombinant baculovirus. The recombinant baculovirus obtained was plaque-purified three times. For large-scale expression, an insect-cell line from *Trichoplusia ni*, BTL-Tn 5B1-4 (Tn5; Invitrogen, San Diego, California, USA), was used and was infected with recombinant baculovirus at a multiplicity of infection of 10. The cells were incubated in EX-CELL-405 medium (JRH Biosciences, Lenexa, Kansas, USA) for 7 d at 300 K. The VLPs were harvested from the supernatant. The recombinant baculovirus and cell debris were removed by centrifugation at 10 000*g* for 90 min at 277 K. The VLPs in the supernatant were then spun down at 100 000*g* for 2 h at 277 K. The resulting VLP pellets were then resuspended in EX-CELL-405 medium and further purified in a CsCl equilibrium density gradient. On inspection by negative-staining EM, the morphology of $VLP_{ORF3/ORF2}$ appeared to be similar to that of VLP_{ORF2}, except for an extra density within the particles.

Prior to crystallization or cryo-electron microscopy (EM) experiments, the purified VLPs were pelleted through a 5%(*w*/*v*) sucrose cushion in 50 m*M* potassium–MES buffer pH 6.2 at 110 000*g* in a Beckman SW 55-Ti rotor at 277 K for 1 h. The pellet was resuspended in 50 m*M* potassium–MES buffer pH 6.2 and maintained at 277 K for 10 min. The concentration of recombinant HEV-VLP was adjusted to 10 mg ml^{-1} according to the standard concentration curve determined from the light absorbance at 260 and 280 nm. The quality of the purified particles was routinely verified by EM using 2%(*w*/*v*) uranyl acetate negative-stain contrast (Agar Scientific Ltd, Stansted, England) and SDS–PAGE performed on 8–25% acrylamide gels under denaturing conditions (Gong *et al.*, 1990; Cheng *et al.*, 1992).

2.2. Cryo-EM and three-dimensional reconstruction of purified $VLP_{ORF3/ORF2}$

Cryo-EM sample preparation followed previously established procedures (Xing *et al.*, 1999). Briefly, a 3.5 µl drop of $VLP_{ORF3/ORF2}$ suspension was applied onto a glow-discharged 'holey' carbon-coated grid, blotted with filter paper and vitrified by rapidly plunging the grids into liquid ethane cooled by liquid nitrogen. The grids, with the frozen $VLP_{ORF3/ORF2}$ physically fixed to fill in the holes of the carbon film after rapid freezing, were transferred to an FEI CM-120 microscope using a Gatan 626DH cryoholder and all subsequent steps were carried out with the sample maintained at 95 K. The electron microscope was operated at 120 kV and low-dose (<7 e^{-} $Å^{-2}$) images were recorded on Kodak SO163 films at a magnification of 45 000×. Selected micrographs with a defocus level of 1000 nm were digitized using a Zeiss microdensitometer (*Z/I* imaging) at a step size of 14 µm, which corresponds to 3.1 Å per pixel at the level of the specimen. The first zero of the contrast transfer function was at a spatial frequency of ~0.056 $Å^{-1}$. Isolated VLP images were extracted from the digitized micrographs, normalized and combined into one single image-stacked file for subsequent processing. Determination of the structure was carried out using a model-based polar Fourier transform (PFT) method (Cheng *et al.*, 1994; Baker & Cheng, 1996). As the PFT algorithm requires a three-dimensional model to start with, a cryo-EM density map of VLP_{ORF2} was used as an initial model (Xing *et al.*, 1999). The model was low-pass filtered to 40 Å resolution in order to reduce the influence of noise bias included in image processing. The starting model was back-projected at 1° angular increments to create an image database that covered all possible views of the model at the orientations within one half of the icosahedral asymmetric unit. Individual unique views of model projections in the database were interpolated onto a polar grid to form a polar projection (PRJ) image and the PRJ image was then Fourier transformed to produce a PFT image; these PRJ and PFT images were stored in two separate files for use as references for alignment with individual images of PFTs and PRJs from the selected VLP projections (Cheng *et al.*, 1994; Baker & Cheng, 1996). In addition, the alignment was performed with enhanced accuracy by initially including a band-pass filter (spatial frequency between 1/90 $Å^{-1}$ and 1/30 $Å^{-1}$) of the PFT images to optimize the search for origins and orientations. A list of origins and orientations corresponding to each particle was obtained and a noise-filtered three-dimensional reconstruction was computed using the Fourier–Bessel algorithm implemented with a cylinder expansion method and imposed 522 symmetry (Crowther, 1971; Cheng *et al.*, 1992; Fuller *et al.*, 1996). The presence of the threefold symmetry in the three-dimensional model validated the accuracy of the reconstruction. The search model was subsequently updated with the newly computed three-dimensional density map of $VLP_{ORF3/ORF2}$ through individual cycles of refinement to make the orientations and origins of the image data to be included in the averaging of the subsequent density maps more accurate. The cryo-EM structural density of $VLP_{ORF3/ORF2}$ for use in initial phasing of the X-ray diffraction data was achieved by the progressive addition of data at higher spatial frequency. The iterations were continued by re-projecting the model at a finer angular increment (0.5°) and by progressively extending the low-pass filter from 30 to 20 Å. The cycles of refinement stopped when no major improvement was observed in the three-dimensional reconstruction. Fourier shell correlations of the reconstruction yielded an estimated resolution of 24 Å for recombinant HEV-$VLP_{ORF3/ORF2}$ based on Fourier averaging of 134 VLP images (Fig. 1). $VLP_{ORF3/ORF2}$ has a diameter of ~270 Å and the capsid shell was composed of 30 dimer-like protrusions arranged in a $T = 1$ icosahedral surface lattice. Analysis of the density-distribution map revealed $VLP_{ORF3/ORF2}$ to consist of 60 subunits of the fusion protein. The VLP structure demonstrated two distinct domains, namely the shell domain, which forms a continuous layer of viral capsid, and the protrusion domain, which forms protruding spikes (Cheng *et al.*, 1992, 1995). The cryo-EM density map of $VLP_{ORF3/ORF2}$ was subsequently used for initial phasing of the data collected by X-ray diffraction.

2.3. Crystallization strategy and data collection

The initial crystallization trials were performed by the hanging-drop method (McPherson, 2004*a*,*b*) with a commercially available kit, Crystal Screen Lite, from Hampton Research (Laguna Niguel, California, USA) at 293 K. The crystallization drops contained 2 µl $VLP_{ORF3/ORF2}$ solution at various concentrations mixed with 2 µl screening solution and were set up for vapor diffusion against 1 ml screening solution in 24-well plates (Falcon). Crystals were obtained using two different conditions: (i) 4%(*w*/*v*) polyethylene glycol (PEG) 4000 in 100 m*M* sodium acetate pH 4.6 and (ii) 4%(*w*/*v*) PEG

8000 in 100 m*M* Tris–HCl pH 8.5. In condition (i) a number of small crystals appeared within a few minutes, while in condition (ii) the crystals appeared after one week. To further assess the integrity of the VLPs packed in the crystals, we selected crystals from both the pH 4.6 and pH 8.5 conditions, dissolved them in the respective reservoir solution and performed negative-staining EM with 2%(*w*/*v*) uranyl acetate (Gong *et al.*, 1990). The VLPs had remained intact within the crystals in both conditions (Fig. 2). The quality of these crystals was assessed using an in-house X-ray generator. The crystals obtained at pH 4.6 diffracted to a lower resolution (40 Å) compared with those obtained at pH 8.5 (20 Å). Based on this result, the crystallization conditions were further optimized by changing the PEG 8000 and VLP concentrations. Good-quality crystals were obtained with 3.5%(*w*/*v*) PEG 8000 in 100 m*M* Tris–HCl pH 8.5; these crystals were rod-shaped and reached a maximum length of 1 mm after 14 d (Fig. 3).

The HEV-VLP$_{ORF3/ORF2}$ crystals were immersed for 2 min in reservoir solution containing 20, 30 or 40%(*v*/*v*) ethylene glycol as a cryoprotectant. A single crystal was picked up with a cryoloop and directly flash-frozen in liquid nitrogen. The HEV-VLP$_{ORF3/ORF2}$ crystals were found to be very fragile and cracked in most cases. Therefore, the VLPs were subsequently crystallized under the same conditions with the addition of 20–40%(*v*/*v*) ethylene glycol to the reservoir. The crystals obtained had a similar appearance to those obtained without the addition of ethylene glycol. One of the resulting crystals was successfully frozen and diffracted X-rays to beyond 7.8 Å resolution at 100 K on a DIP6040 imaging-plate/CCD hybrid detector (MacScience, Bruker-AXS) with a crystal-to-detector distance of 700 mm, an oscillation angle of 1.0° and an exposure time of 10 s using synchrotron radiation at SPring-8 (Hyogo, Japan) beamline BL44XU (Fig. 4). As the crystal decayed during data collection, the data set was only processed to 8.3 Å resolution. The diffraction images were indexed, reduced, scaled and merged using the *HKL-2000* package (Otwinowski & Minor, 1997). The intensities were converted into the structure-factor amplitudes using *TRUNCATE* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). The space group was determined to be $P2_12_12_1$ by scaling in the resolution range 70–8.3 Å assuming Laue group 222 (R_{merge} = 13.6% from *SCALEPACK*) and was assigned on the basis of systematic absences of odd reflections along the *h*00, 0*k*0 and 00*l* axes. The unit-cell parameters were a = 337, b = 343, c = 346 Å, $\alpha = \beta = \gamma = 90°$. The statistics of data collection are summarized in Table 1. The value of $I/\sigma(I)$ was found to be 8.3 and 2.2 for the resolution ranges 70–8.3 and 8.6–8.3 Å, respectively. The R_{merge} for the outermost resolution shell was slightly worse than for most low-symmetry protein structure determinations. In this case of viral crystallography, the additional noncrystallographic averaging (60-fold) and the solvent flattening provided the phasing power required to successfully employ the diffraction data to 8.3 Å resolution. While four particles were found in one unit cell (with a molecular weight of 3.2 × 10^6 Da), there is only one complete VLP per

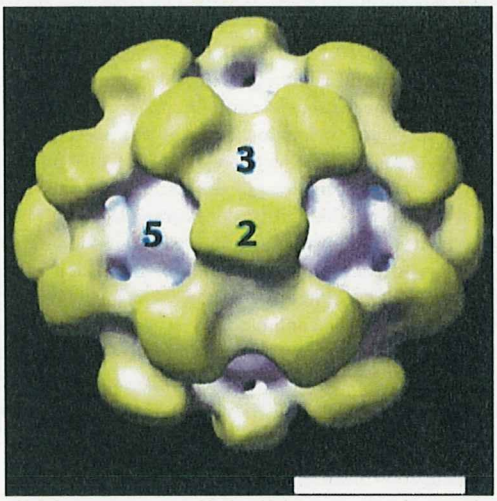


Figure 1
Three-dimensional structure of recombinant HEV-VLP$_{ORF3/ORF2}$ at a resolution of 24 Å determined by cryo-EM and image reconstruction. An isosurface representation of the outer surface of recombinant HEV-VLP$_{ORF3/ORF2}$ is shown viewed along the icosahedral twofold axis. The surface density was contoured at a level corresponding to 100% mass of the expected particle volume. The particle is color-coded to differentiate two distinct domains: the shell domain (white) and the protrusion domain (yellow). The surface capsid conforms to T = 1 icosahedral symmetry in which the 60 subunits are arranged into 30 protruding spikes with the homodimers as the basic building blocks at each icosahedral twofold axis. The scale bar represents 100 Å.

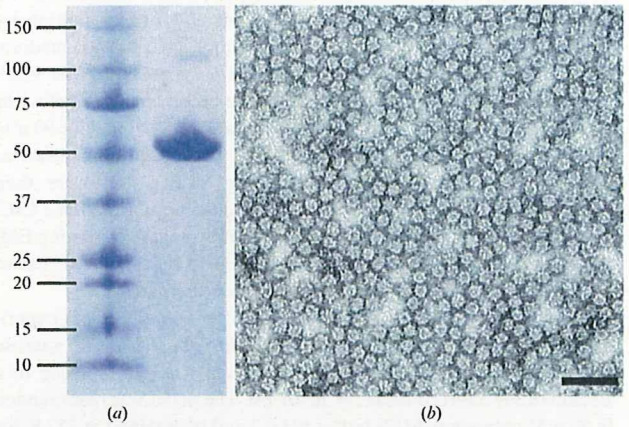


Figure 2
Purity and integrity of recombinant HEV-VLP$_{ORF3/ORF2}$ crystals grown in 4%(*w*/*v*) PEG 8000, 100 m*M* Tris–HCl pH 8.5 analyzed by SDS–PAGE (*a*) and negative-stained electron microscopy (*b*). In (*a*), the left lane contains molecular-weight markers (kDa) and the right lane contains the protein band of recombinant HEV-VLP from a dissolved crystal. In (*b*), a recombinant HEV-VLP$_{ORF3/ORF2}$ crystal was crushed with a nylon loop and stained with 2% uranyl acetate; the VLPs remained with intact capsid morphology after dissolving from the crystal. The bar represents 1000 Å.

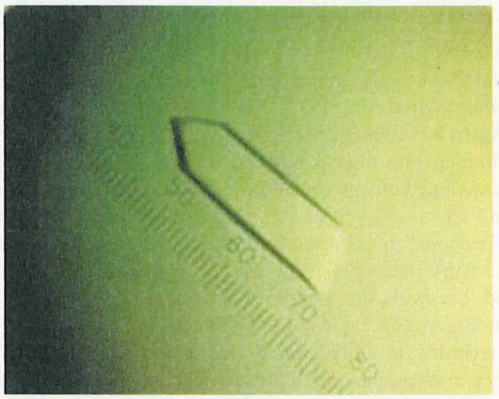


Figure 3
Recombinant HEV-VLP$_{ORF3/ORF2}$ crystal. The crystal was grown in Tris–HCl pH 8.5 buffer, 3.5%(*w*/*v*) PEG 8000 with the addition of 30% ethylene glycol as a cryoprotectant. In the scale, 28 intervals represent 0.1 mm.

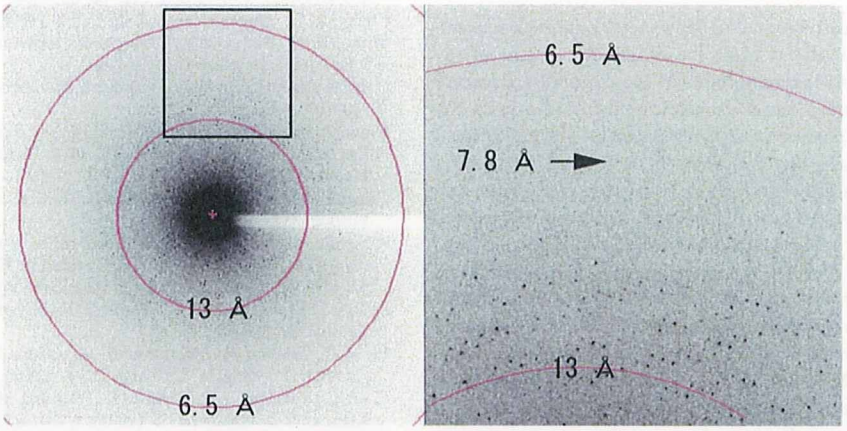
asymmetric unit, resulting in 60-fold noncrystallographic symmetry redundancy. The calculated Matthews coefficient V_M is 3.1 Å^3 Da^{-1} (Matthews, 1968).

2.4. Phase determination

A self-rotation function was computed using the program *POLARRFN* from the *CCP*4 package in order to determine the orientation of the icosahedral noncrystallographic symmetry elements. By using reflections in the resolution range 15–10 Å, a fast rotation function was calculated with an integration radius of 130 Å and a *B* factor of −70 Å^2. The section corresponding to the fivefold axis is depicted in Fig. 5(*a*). The fivefold rotation function was consistent with the presence of four particles per unit cell. Six peaks were clearly identified corresponding to one of the four particles in the unit cell. While additional peaks were observed corresponding to the symmetry-related particles, some unexpected peaks might arise from the 72° rotational relationship between the icosahedral particles, as they were reproduced from the calculated data using a cryo-EM map. Subsequently, the molecular-replacement method starting from a cryo-EM density map (Fig. 1) was used to phase the reflections. The original cryo-EM map was rotated to superimpose the icosahedral symmetry axes of the cryo-EM density onto the VLP orientation determined by the rotation function using the matrix

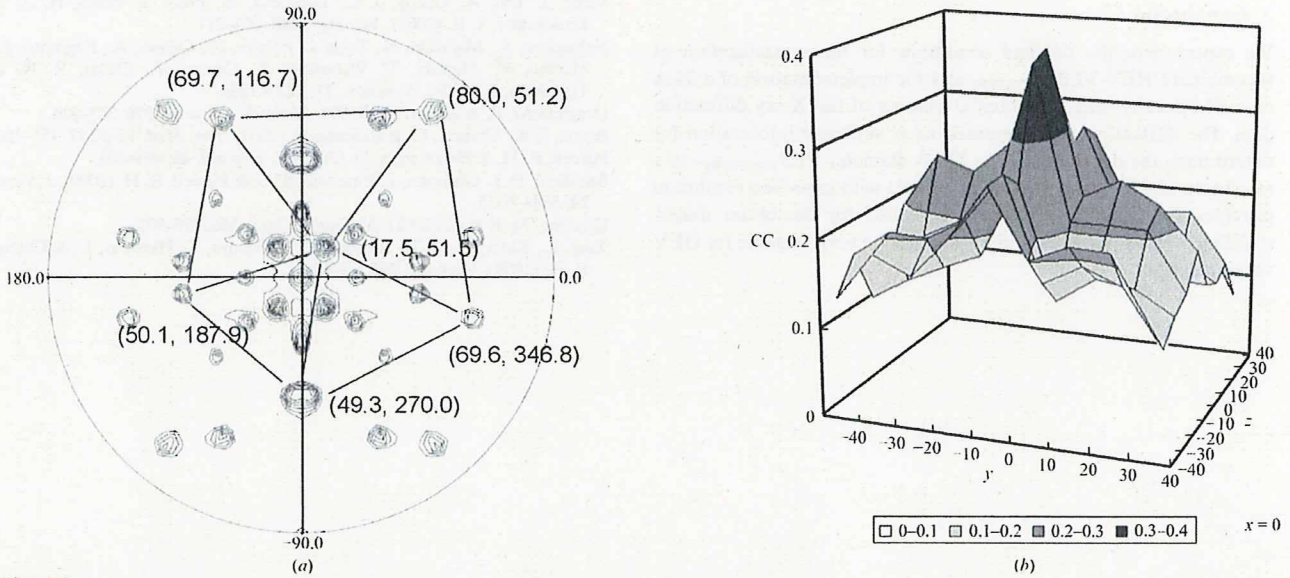
$$\begin{pmatrix} 0.901271 & -0.269099 & -0.339552 \\ 0.311789 & 0.947022 & 0.077054 \\ 0.300829 & -0.175315 & 0.937426 \end{pmatrix}.$$

Packing considerations suggested that the particle is situated close to the positions in space group $P2_12_12_1$ with $x = 0$, $y = 0$, $z = 0$ or $x = 0.25$,



(*a*) (*b*)

Figure 4
A diffraction pattern recorded from a recombinant HEV-VLP$_{ORF3/ORF2}$ crystal. (*a*) A typical 1.0° oscillation photograph exposed for 10 s. The concentric circles indicate the 13.0 and 6.5 Å resolution shells. (*b*) An enlarged image shows a diffraction spot observed at 7.8 Å (indicated by an arrow).



(*a*) (*b*)

Figure 5
Phasing X-ray data with a cryo-EM density map. (*a*) Fivefold self-rotation function peaks calculated in *POLARRFN* with data in the resolution range 15–10 Å and a radius of integration of 130 Å. The positions (φ, ψ) corresponding to one of the four particles in the unit cell are indicated. (*b*) Translational correlation coefficient search for the origin of recombinant HEV-VLP$_{ORF3/ORF2}$. The data used in the calculations were in the resolution range 70–30 Å (only the result at $x = 0$ is shown). The search grid started at the coarse interval of 10 Å and was refined at a finer interval of 2 Å. The maximum correlation coefficient was observed at the point (−2, 0, −6 Å).

Table 1
Crystal information and data-processing statistics.

Values in parentheses are for the outermost shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	$a = 337$, $b = 343$, $c = 346$, $\alpha = \beta = \gamma = 90$
Resolution range (Å)	70.00–8.30 (8.60–8.30)
Wavelength (Å)	0.9
Total No. of crystals	1
Total No. of reflections	101760
Unique reflections	33414 (3347)
Completeness (%)	87.6 (89.4)
Multiplicity	3.0 (3.0)
$I/\sigma(I)$	8.3 (2.2)
R_{merge}†	0.136 (0.500)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity for all measurements including symmetry equivalents.

$y = 0.25$, $z = 0.25$. The correlation coefficient was computed between the structure factors observed from the amplitudes of the X-ray diffraction data (F_o) and the amplitudes of the calculated structure factor (F_c) derived from Fourier transformation of the cryo-EM model properly rotated and positioned in the crystal unit cell around these two positions using the programs *MAVE* from the *RAVE* package of the Uppsala Software Factory (Kleywegt *et al.*, 2001) and *SFALL* and *RSTATS* from the *CCP*4 package. The translation search was initially carried out with a coarse interval of 10 Å steps using data in the resolution range 70–30 Å. After searching with a finer interval of 2 Å, a maximum correlation coefficient value of 0.41 was reached at the origin (−2, 0, −6 Å) (Fig. 5*b*). Phase refinement and extension were carried out in the resolution range 30–8.3 Å using real-space averaging and solvent flattening with the *RAVE* and *CCP*4 packages as performed in our previous work (Nakagawa *et al.*, 2003). The final correlation coefficient and R factor between the F_os and the F_cs obtained from inversion of the averaged and solvent-flattened map at 8.3 Å resolution were 0.92 and 0.21, respectively.

3. Conclusion

We report here the detailed conditions for the crystallization of recombinant HEV-VLP$_{ORF3/ORF2}$ and the implementation of a 24 Å cryo-EM density map in the initial phasing of the X-ray diffraction data. The diffraction data presented carry sufficient information for determining the density map of a 270 Å diameter VLP$_{ORF3/ORF2}$ to a resolution of 8.3 Å. The availability of data with improved resolution provides the structural information needed for the better understanding of virus-particle assembly and will be very valuable for HEV vaccine design.

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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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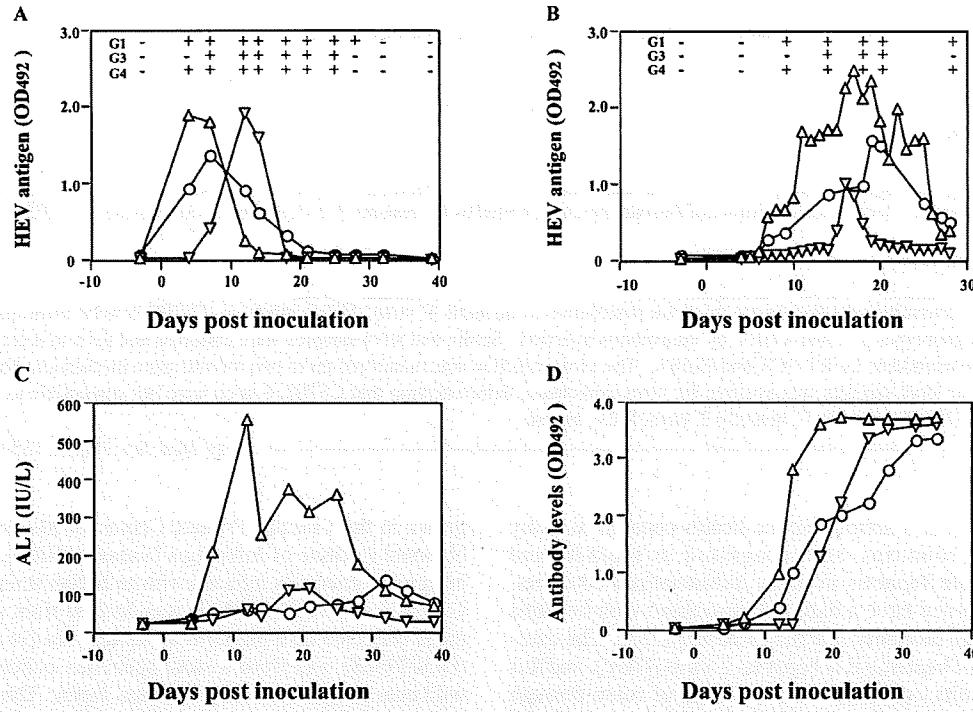

Fig. 1. Kinetics of biochemical, serological, and virological markers in monkeys after inoculation. HEV-Ag and HEV RNA in sera (A) and stools (B), was measured by an antigen ELISA and RT-PCR. ALT was indicated (C), and IgG antibody was measured by an antibody-ELISA (D). ○, monkey inoculated with G1 HEV;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