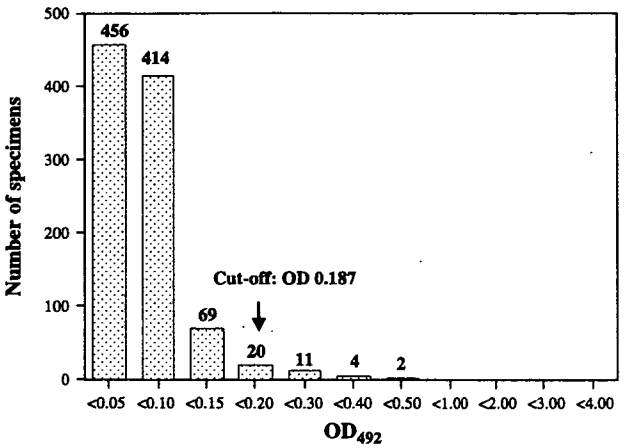

Fig. 2. Distribution of OD values of IgG antibodies. Serum samples from 976 deer were tested by ELISA. The arrows indicate the cutoff values

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

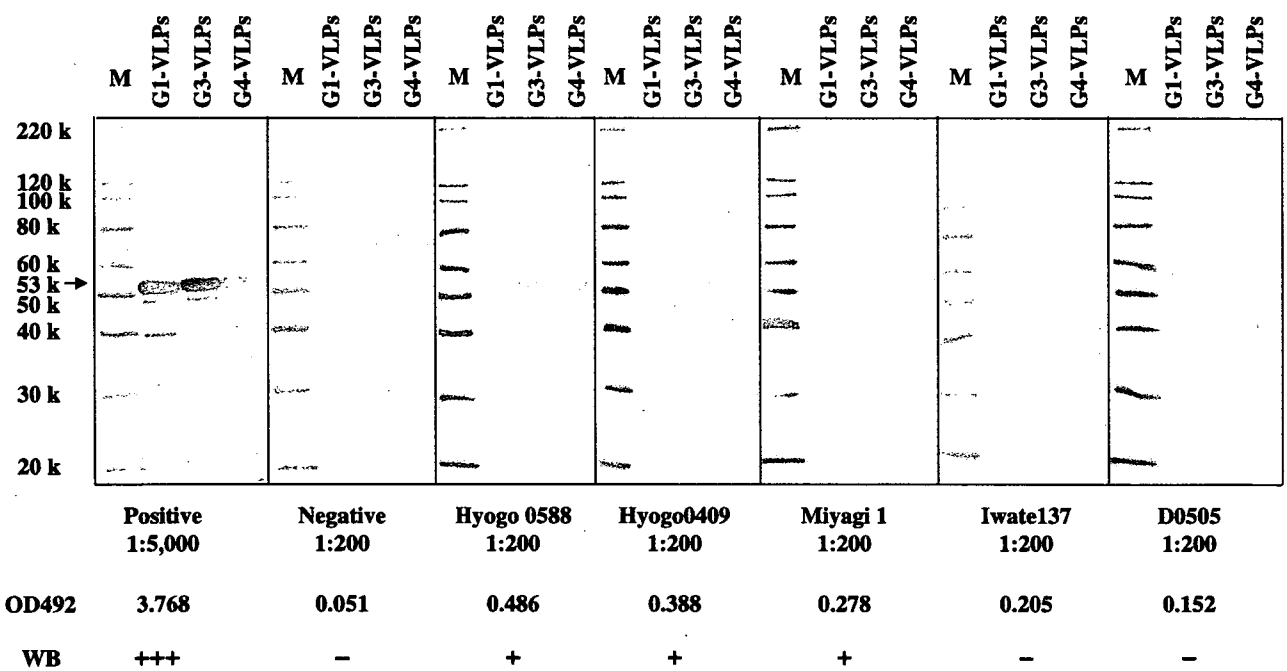

Fig. 3. Specificity of the IgG antibody determined by Western blot assay. The G1, G3, and G4 VLPs were used as the antigens, and 7 deer sera with different OD values were evaluated. The results of the Western blot assay are indicated as +++ (strong band), + (weak band), or – (no band). *M* Molecular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

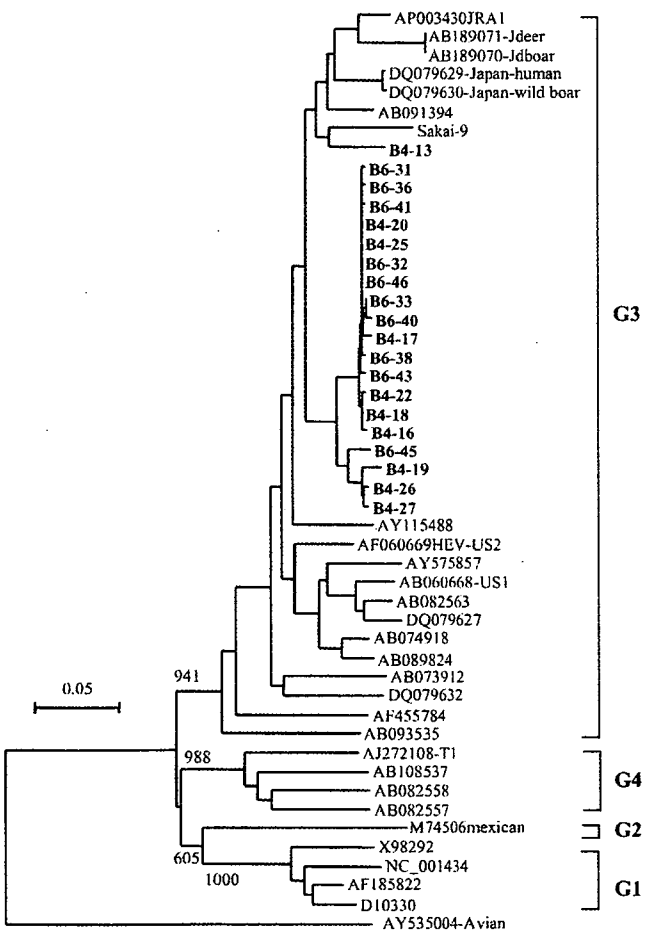

FIGURE 1. Phylogenetic trees of hepatitis E virus (HEV) constructed with avian HEV as an outgroup. A partial open reading frame 2 ORF2 (338 basepairs) of each of 10 clones of B4 (B4-13, B4-16, B4-17, B4-18, B4-19, B4-20, B4-22, B4-25, B4-26, and B4-27) and B6 (B6-31, B6-32, B6-33, B6-36, B6-38, B6-40, 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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