Table 2 Anti-HEV in the wild-caught boar in relation to year and area of capture

Year of capture	Area of capture				Total
	East	Central	South-west	Unknown	
2001	1/38 (2.6%)	10/30 (33.3%)	3/27 (11.1%)	1/10 (10.0%)	15/105 (14.3%)
2002	2/15 (13.3%)	3/50 (6.0%)	1/27 (3.7%)	1/4 (25.0%)	7/96 (7.3%)
2003	4/21 (19.0%)	24/44 (54.5%)	0/3 (0%)	0/0 (0%)	28/68 (41.2%)
2004	5/42 (11.9%)	38/64 (59.4%)	7/15 (46.7%)	0/2 (0%)	50/123 (40.7%)
Total	12/116 (10.3%)	75/188 (39.9%)	11/72 (15.3%)	2/16 (12.5%)	100/392 (25.5%)

(Table 3). An 821-nt sequence was determined in 10 of the 12 HEV-RNA-positive samples (the other two samples were unable to sequence the 821-nt, but a shorter sequence, 69-nt, was available from them) for phylogenetic analyses. As shown in Figure 2a, all the wild boar-derived 821-nt sequences analyzed in this study segregated to genotype 3 (those for which only 69-nt was available were also of genotype 3), but were clearly distinct from any of the previously reported sequences. Interestingly, eight isolates from the wild boar in this study conformed to a compact cluster. This was conceivable because all of the eight isolates were derived from boar living in the same habitat within the studied area. The other two isolates, genetically distant from the eight isolates, were from boar captured at different sites (Fig. 2b).

The positive rates of anti-HEV showed a significant difference between wild-caught boar and bred boar in total (25.5% vs. 71.4%, $P < 0.001$), as well as in an age-matched comparison: anti-HEV was positive in 18.2% (14/44) of the wild-caught, but in 69.2% (9/13) of the bred boar ($P < 0.05$) when only those aged 1–2 years were compared. Despite quite a high rate of anti-HEV, none of the bred boar was positive for HEV-RNA, similarly to the reported case of bred swine in which viremia was recognized only within 6 months of birth.[36]

DISCUSSION

OUR PRESENT RESULTS showed that approximately 25% (100/392) of boar caught in the wild were positive for anti-HEV, with HEV-RNA in 3% (12/392).

Table 3 The boar positive for HEV-RNA

Age (years)	Sex	Place	Year	Anti-HEV (OD)	Genotype	HEV isolate name
0–1	F	South-west	2003	(-)	3	JBOAR221-Ehi03R
1–2	F	Central	2001	3.010	3	‡
1–2	F	Central	2003	2.938	3	JBOAR247-Ehi03R
1–2	M	Central	2003	2.366	3	JBOAR243-Ehi03R
1–2	F	Central	2004	1.451	3	JBOAR288-Ehi04R
2–3	F	Central	2003	3.000	3	JBOAR261-Ehi03R
2–3	F	Central	2003	2.723	3	JBAOR263-Ehi03R
2–3	M	East	2004	(-)	3	JBOAR351-Ehi04R
†	M	South-west	2002	1.557	3	JBOAR024-Ehi02R
†	†	East	2002	2.175	3	‡
†	F	Central	2004	3.220	3	JBOAR300-Ehi04R
†	†	South-west	2004	(-)	3	JBOAR398-Ehi04R

†Unknown; ‡Only a 69-nt sequence was available.
OD, optical density.

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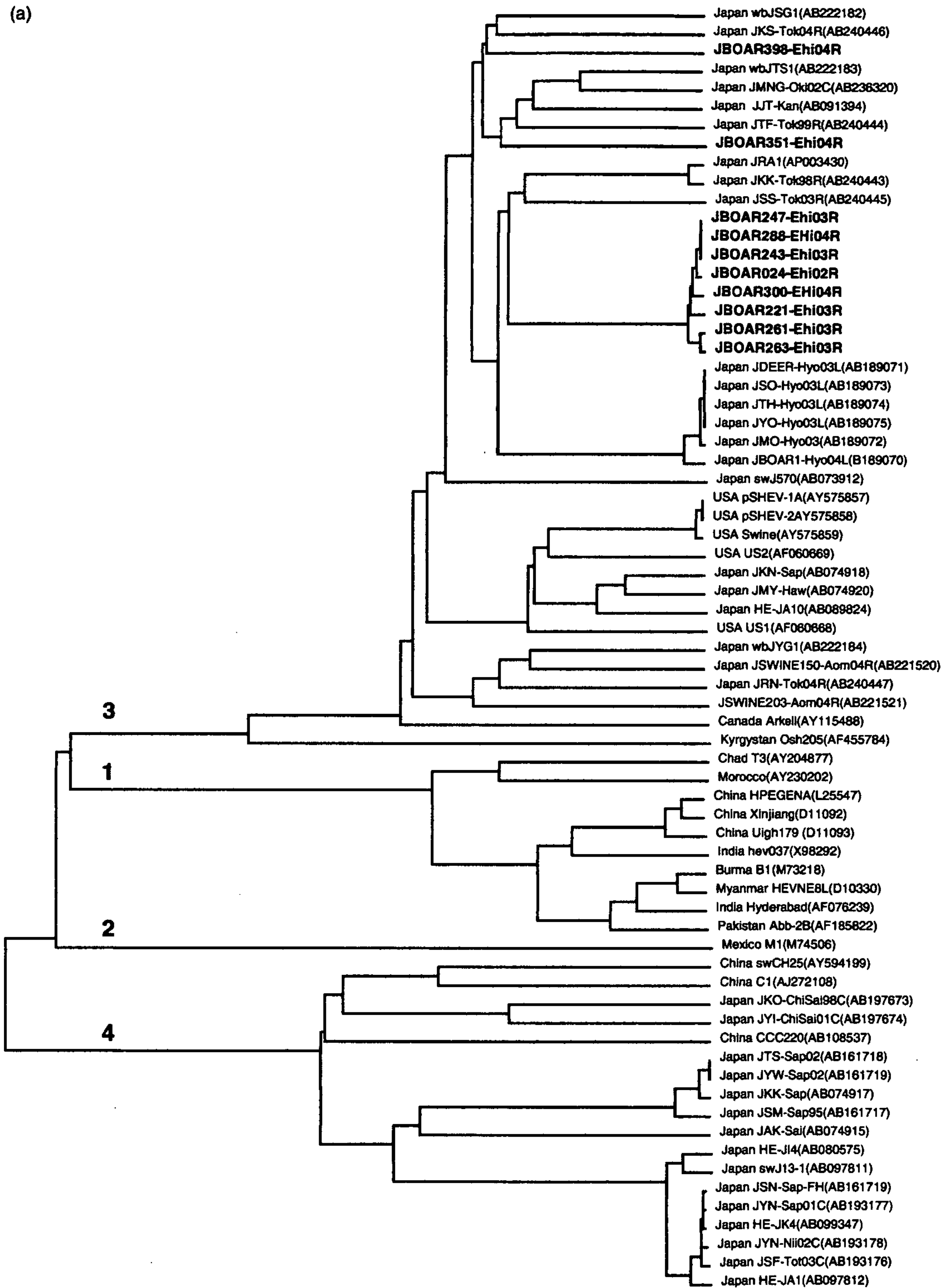

Figure 2 (a) Phylogenetic tree based on the partial nucleotide sequence of ORF1 (821-nt). DDBJ/EMBL/GenBank accession numbers are shown in parentheses.

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(b)

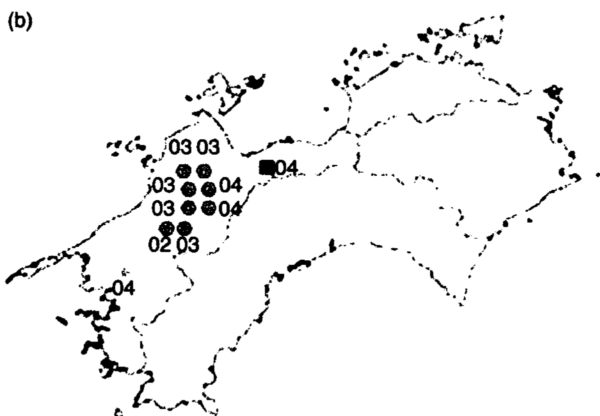

Figure 2 *Continued.* (b) Information on the place and year of capture for the 10 wild boar from which the 821-nt sequence was available. Circles, squares, and stars correspond to the isolates similarly highlighted in Figure 2(a). Numbers shown in the figure represent the year in which the boar was caught (02, 03, and 04 represent the years 2002, 2003, and 2004, respectively).

In the present study, three of 12 HEV-RNA-positive boar were negative for anti-HEV, which may suggest that these three were infected with HEV very recently. The rate of viremic boar is striking, because HEV infection is basically transient, not persistent, which would allow viremia be detected much easier. Our findings indicate that HEV is endemic in wild boar, at least in the Ehime area of Japan. We have a patient with acute hepatitis E in this area who had a history of cooking and eating the meat of wild boar (unpublished data), therefore wild boar should be regarded as an important infectious source.

Wild boar are distributed throughout Europe, Africa, Asia and some areas of North America, and their habitat includes broad-leaved woodland and grassland areas. They eat both plants and small animals, such as roots, fruits, nuts, insects and small mammals. They often live near muddy puddles where they routinely wallow so as to remove parasites from their body surface. Female wild boar remain with their offspring and form a family group, whereas older males live alone. During the mating season in autumn, several family groups often live together and form a herd. Although the transmission routes of HEV among wild boar remain unknown, it is possible that their habit of wallowing in mud and their tendency to form herds may have some association with fecal–oral transmission.

As for the bred boar, in which quite a high rate of anti-HEV was found in this study, their situation is more comparable to farm pigs than to the boar in the wild. They are kept together in a limited space with a limited freedom, this means the horizontal spread of infective agents occurs more easily than in the wild. In such circumstances HEV infection occurs very early on: it has been reported that pigs kept in farms get infected with HEV within a few months of birth.[36] The same thing may have happened in the bred boar population studied here. In the same way that most farm pigs lost HEV-RNA before 6 months of age, our bred boar may have lost it by the time they were subjected to this study (all were aged more than 1 year).

HEV is classified into four major genotypes[37] with different distribution by geographic regions: genotype 1 in Asia and Africa, genotype 2 in Mexico and Nigeria, genotype 3 in the USA, European countries, Argentina, Korea and Japan, and genotype 4 in east Asian countries.[38–45] All of the 12 HEV-RNA-positive wild boar in the present study had HEV of genotype 3. In other studies in Japan, genotypes 3 and 4 HEV have been isolated from both humans and boar.[25,30,31,34] Being the same genotype as those previously reported from Japan notwithstanding, the HEV isolates we report here are phylogenetically unique, as shown in Figure 2a. In addition, the finding that there were three different strains (one cluster consisting of eight isolates, and two solitary strains) corresponding to three different habitats might reflect the fact that wild boar tend to establish their own territory and hardly leave that area.

In conclusion, the results of the present study of wild boar captured in the Ehime area suggest that the wild boar in this area are an important reservoir of HEV, and should be regarded as an infectious source for humans.

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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

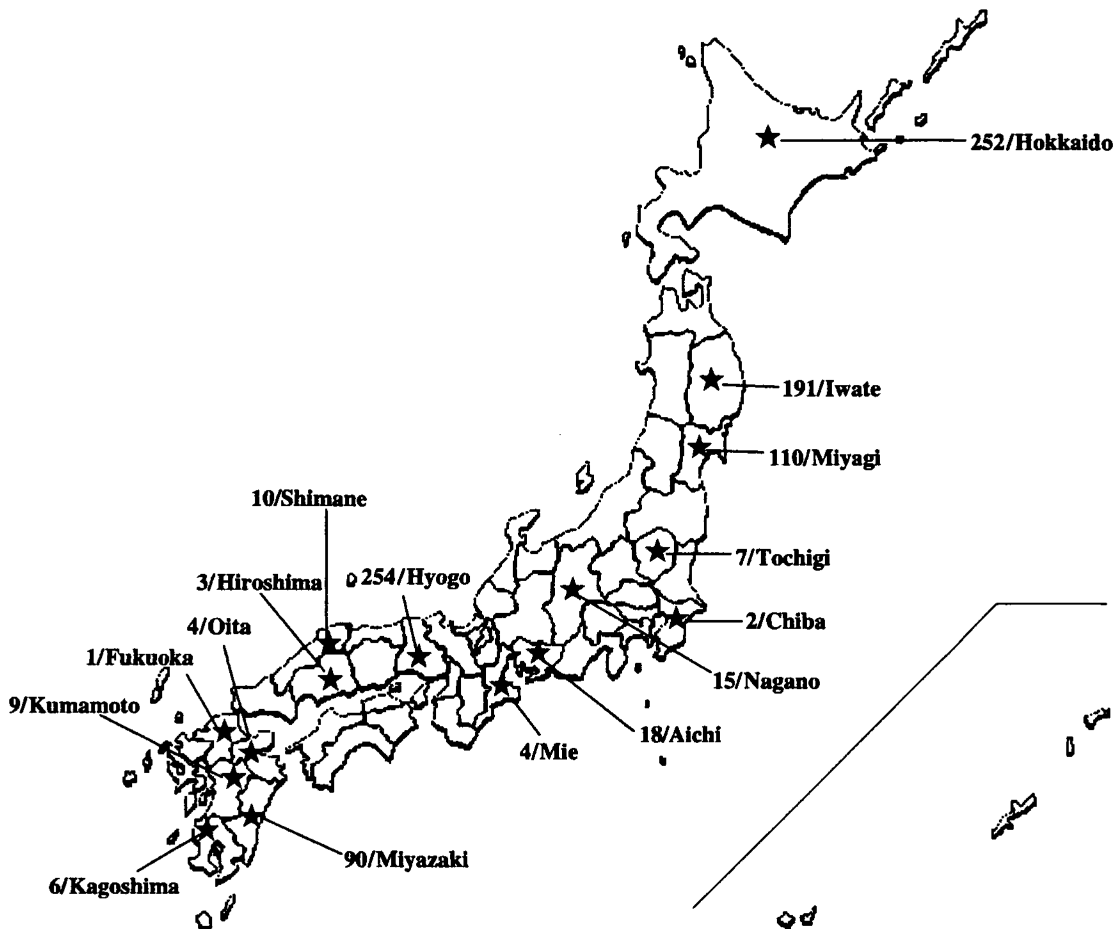

Fig. 1. Numbers and prefecture of captured wild sika deer

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.

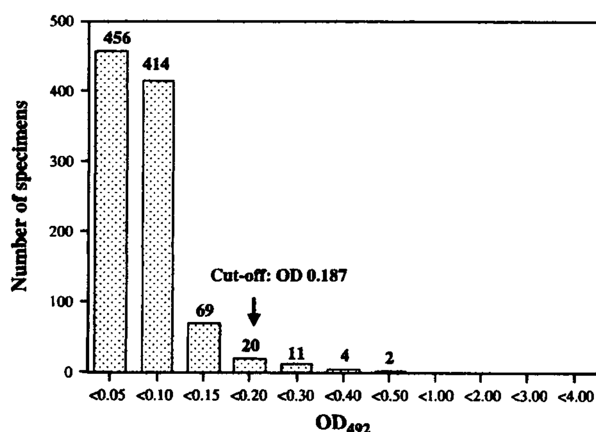

Fig. 2. Distribution of OD values of IgG antibodies. Serum samples from 976 deer were tested by ELISA. The arrows indicate the cutoff values

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

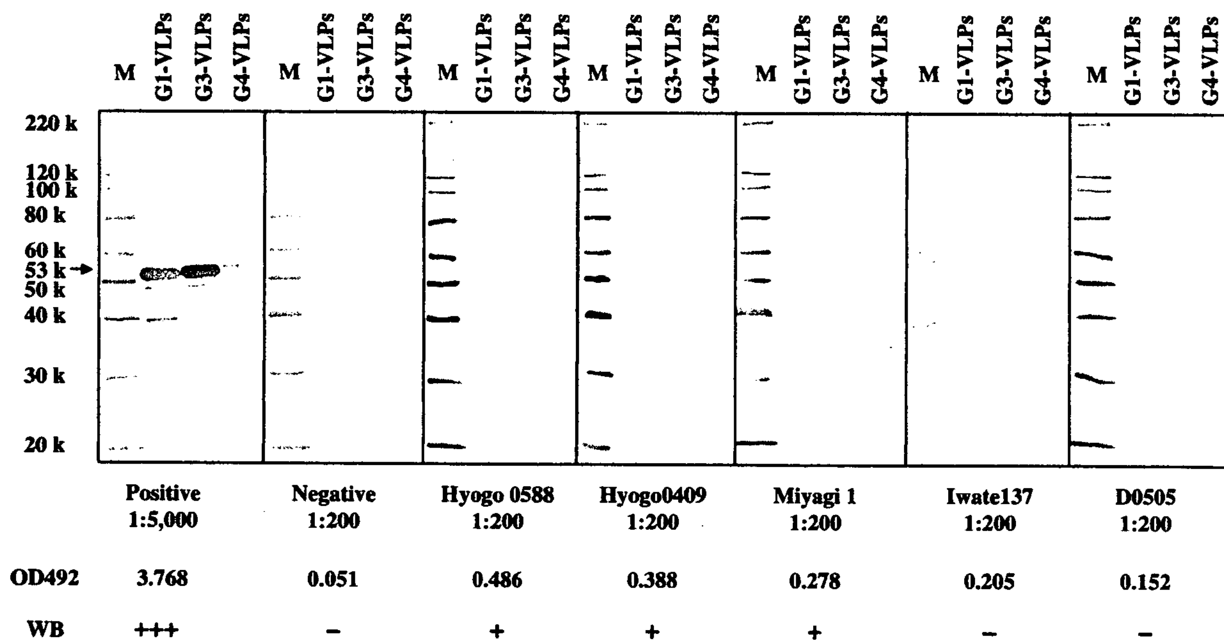

Fig. 3. Specificity of the IgG antibody determined by Western blot assay. The G1, G3, and G4 VLPs were used as the antigens, and 7 deer sera with different OD values were evaluated. The results of the Western blot assay are indicated as +++ (strong band), + (weak band), or – (no band). *M* Molecular 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^{TM} II RNase H^- reverse transcriptase (Invitrogen, Inc., Carlsbad, CA), 1 μl of the oligo (dT) primer, 1 μl of RNaseOUT^{TM}, 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′-TGYTGGTTRTCRTARTCCTG-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′-TAYCGHAAYCAAGGHTGGCG-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.

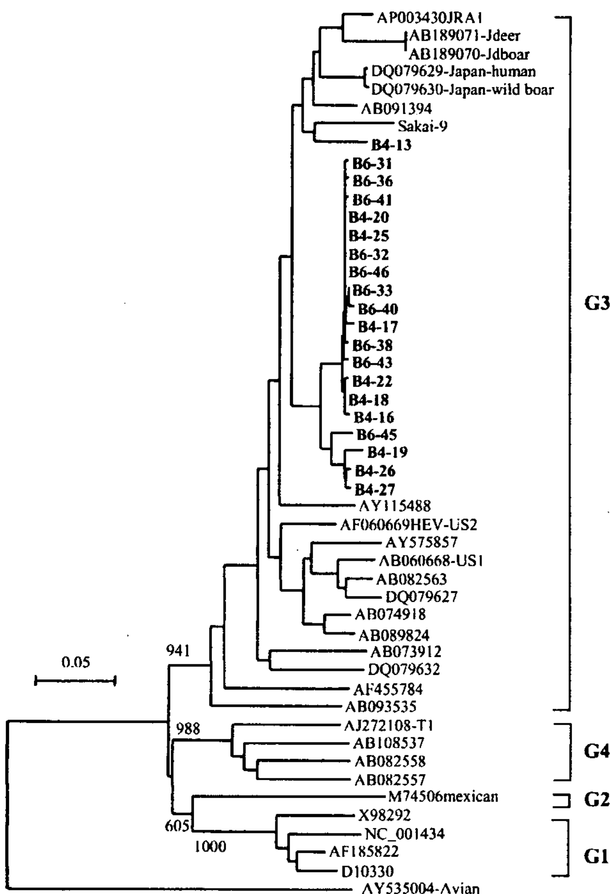

FIGURE 1. Phylogenetic trees of hepatitis E virus (HEV) constructed with avian HEV as an outgroup. A partial open reading frame 2 ORF2 (338 basepairs) of each of 10 clones of B4 (B4-13, B4-16, B4-17, B4-18, B4-19, B4-20, B4-22, B4-25, B4-26, and B4-27) and B6 (B6-31, B6-32, B6-33, B6-36, B6-38, B6-40,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

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HEPATITIS E virus (HEV) is the causative agent of human hepatitis E in many developing and some industrialised countries (Aggarwal and Krawczynski 2000, Meng 2000a, Schlauder and Mushahwar 2001, Huang and others 2002, Mizuo and others 2002, Takahashi and others 2003). It is widely known that in humans HEV is primarily transmitted by the faecal-oral route through contaminated water, and occasionally causes large epidemics in endemic areas (Arankalle and others 1994, Aggarwal and Krawczynski 2000). However, in non-endemic areas, the reservoir for sporadic human cases with no history of travel to HEV-endemic areas has remained unknown for many years (Harrison 1999, Schlauder and Mushahwar 2001, Mizuo and others 2002, Takahasi and others 2002). In 1997, the first animal HEV strain, swine HEV, was discovered in a pig in the USA (Meng and others 1997). Subsequent epidemiological studies indicated that, for example, in Japan, most farmed pigs had been exposed to swine HEV (Takahashi and others 2003). There is a growing consensus that HEV is a potential zoonotic agent and that pigs can act as a reservoir for humans.

HEV has also been detected in chickens (Haqshenas and others 2001) and wild rodents (Kabrane-Lazizi and others 1999, Favorov and others 2000, Arankalle and others 2001, He and others 2002, Hirano and others 2003a), and anti-HEV antibodies have been found in cattle, water buffaloes, sheep, goats, non-human primates, dogs and cats (Tsarev and others 1993, Meng 2000b, Arankalle and others 2001, Hirano and others 2003b, Usui and others 2004). In an unusual case, an incident was reported in which a cat was suspected to be a reservoir for human infection (Kuno and others 2003). This short communication describes the examination of blood samples and rectal swabs from dogs and cats for evidence of HEV infection.

An ELISA using the purified, empty virus-like particles (VLPs) of HEV (Li and others 1997) was performed with 424 canine and 202 feline serum samples. Samples were collected from animals at animal hospitals over a five-year period, from 2000 to 2004, in over 30 prefectures covering northern Hokkaido and south-western Okinawa, Japan, and stored at –20°C. The ELISA method used was the same as that described by Li and others (2000). The sera were examined simultaneously using VLP-coated and VLP-uncoated (blank) plates, and the antibody titre was expressed as an optical density at 492 nm (OD_{492}) by subtracting the OD_{492} value of the blank plate from that of the VLP-coated plate.

OD_{492} values of greater than 0·1 were obtained from 10 canine and eight feline serum samples (Table 1). These samples were subsequently absorbed with the same VLPs used as the antigen in the ELISA to confirm the specificity of the reaction. The serum sample (100 µl at a dilution of 1:200) was mixed with 1 µg of VLPs, and the mixture was incubated at 37°C for one hour, and then re-examined. A reduction of the OD_{492} value of the sample by greater than 50 per cent after the absorption was considered to indicate an anti-HEV antibody-positive result, as described by Arankalle and others (2001). On this basis all the canine serum samples were considered to be anti-HEV antibody-negative, and four feline serum samples (1·98 per cent) were considered to be anti-HEV antibody-positive (Table 2).

A reverse transcriptase-PCR (RT-PCR) assay, described for the detection of swine HEV (Huang and others 2002), was used to test 100 canine and 66 feline rectal swabs from animal hospitals located in Tokyo and its environs obtained over a six-year period from 1999 to 2004. In addition, the 18 sera showing an OD_{492} value of greater than 0·1 (Table 1) were also examined by RT-PCR. When a questionable PCR product was obtained, it was analysed by sequencing. No specific PCR products were amplified from any of the canine or feline samples examined.

Only a small number of cases of HEV infection in dogs and cats have been reported (Tien and others 1997, Arankalle and others 2001, Usui and others 2004). In the present study, the samples were collected nationwide in Japan and anti-HEV antibody was found in a very small percentage of the cats and in none of the dogs tested by the ELISA. These results were in contrast to a previous report. Among 135 cats visiting an animal hospital in a provincial capital in Japan, 44 (33 per cent) possessed anti-HEV antibody but no HEV RNA was recovered from the 135 sera (Usui and others 2004). No convincing explanation for the discrepancy in the seroprevalence rates between the previous and the present reports was possible. It should be noted that there is a possibility that the recombinant antigen itself could have non-specific, cross-reacting epitopes among antibodies in animal sera. According to a report describing HEV infection in dogs from India (Arankalle and others 2001) and Vietnam (Tien and others 1997), 22·7 per cent to 27 per cent of the dogs were anti-HEV antibody-positive. It may be true that dogs and cats in such HEV-endemic areas have been exposed to HEV more frequently than animals in non-endemic areas.

In non-endemic areas, there may be some common infectious source affecting both human beings and their companion animals, as they share mostly the same living environment. One probable reservoir, especially for companion animals, is rodents, and it has recently been reported in Japan that wild rats have been found to be infected with HEV (Hirano and others 2003a). Cats are rodent hunters, which is inherent to their nature, but this is not so much the case in dogs. This causal relationship between disease prevalence and a behavioural characteristic has already been seen in other viral diseases in cats, such as Borna disease virus (Berg and

TABLE 1: Results of an ELISA for antibody to hepatitis E virus in dog and cat sera from Japan

Animal	Number of samples tested	Optical density (mean) at 492 nm ≤0	0·001-0·099	0·1-0·499	≥0·5
Dog	424	254	160 (0·039)	10 (0;113)	0
Cat	202	83	111 (0·044)	7 (0·178)	1 (1·189)

TABLE 2: Details of cats suspected or confirmed as hepatitis E virus (HEV) antibody-positive by ELISA

Cat	Place of residence	Age (years)	Sex	Clinical signs	Reduction rate of ELISA optical density at 492 nm after absorption (%)	HEV antibody*
1	Niigata	Unknown	MN	None	60	+
2	Tokyo	11	MN	Kidney dysfunction	49·6	Suspected
3	Tokyo	6	F	None	58·6	+
4	Kanagawa	7	M	Stomatitis	32·3	Questionable
5	Aichi	15	FN	None	82·1	+
6	Okinawa	2-3†	M	None	96·2	+

* Greater than 50 per cent reduction was regarded as HEV antibody-positive
† Estimated
MN Male neutered, F Female, M Male, FN Female neutered, + Positive

others 1998) and cowpox virus infections (Nowotny and others 1994).

In conclusion, natural HEV infections in both dogs and cats in Japan appear to be rare and these animal species may be accidental hosts for human beings.

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Influence of food intake on the clinical response to cyclosporin A in canine atopic dermatitis

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CYCLOSPORIN A (CsA) (Atopica, Atoplus soft capsules; Novartis Animal Health) was introduced recently as an oral microemulsion formulation for the treatment of dogs with atopic dermatitis. Several randomised, controlled and open clinical studies have proven its clinical efficacy (Olivry and others 2002, Steffan and others 2003, 2004). Based on pharmacokinetic data, administration of CsA is recommended two hours before or after feeding, as its bioavailability decreases and the variability of individual blood concentrations increases when the drug is given with food (Steffan and others 2004). Dosing fasted dogs is therefore recommended to optimise the drug's bioavailability. However, the clinical response of dogs after dosing with or without food has never been evaluated. This short communication describes a study to determine whether the administration of CsA with or without food could influence the clinical response in dogs with atopic dermatitis.

Twenty-five dogs with non-seasonal atopic dermatitis, diagnosed by Prélaud's criteria (Prélaud and others 1998) and by exclusion of differential diagnoses, were included in a multicentre, randomised study involving seven investigators

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Case Report

A case of acute hepatitis E associated with multidrug hypersensitivity and cytomegalovirus reactivation

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A 65-year-old Japanese man was hospitalized because of acute hepatitis and severe cholestasis due to hepatitis E virus (HEV) infection combined with a drug reaction to a cold preparation. He died of disseminated intravascular coagulation and severe intestinal bleeding due to systemic cytomegalovirus reactivation following the development of severe eruptions with marked eosinophilia due to drug hypersensitivity to taurine and ursodeoxycholate preparations. The close interaction between viral infection or reactivation and drug hypersensitivity was considered as a pathophysiology in this case, which emphasizes the need for further study of the immunological mechanism of the interaction.

Key words: cholestasis, drug hypersensitivity, eosinophilia, eruption, hepatitis E, hypersensitivity syndrome

INTRODUCTION

SOME VIRAL SPECIES, such as the Epstein–Barr (EB) virus,[1–3] induce drug hypersensitivity associated with eruptions. Cases of severe eruptions caused by viral reactivation, usually by human herpesvirus-6 (HHV-6), induced by primarily occurring drug hypersensitivity, have recently been reported and designated as hypersensitivity syndrome (HS).[4] The association between viral infection or reactivation and drug allergy is therefore a major area of concern in studying the immunological mechanism of hypersensitivity. Here, we report a case demonstrating multidrug hypersensitivity and cytomegalovirus reactivation following acute hepatitis E virus (HEV) infection.

CASE REPORT

THE PATIENT WAS a 65-year-old Japanese man. He took a commercially available medicine for a common cold, Jikinin, because of his rhinorrhea and coughing in the middle of February, 2004. He noted dark urine and pruritus of the whole body on 1 March, and visited Iwate Prefectural Ohfunato Hospital on 9 March. He was hospitalized on the day of his visit with a diagnosis of acute hepatitis from the clinical findings of overt jaundice and elevated levels of liver enzymes (Table 1). The laboratory data obtained at this stage demonstrated acute hepatic injury with cholestasis, but without any sign of hepatic failure. He was then transferred to the Iwate Medical University Hospital on 12 March because of further elevation in the levels of serum bilirubin and liver enzymes.

On admission, he showed marked jaundice on the bulbar conjunctiva and skin, but no abnormality in consciousness and vital signs. Laboratory findings showed a marked increase in serum bilirubin level and a moderate increase in the levels of liver enzymes, but no abnormality in total protein concentration, albumin level or blood coagulation test results (Tables 1 and 2). Leukocyte bands showed no abnormal classification, and no eosinophilia was found at this stage. Serological screening tests for viral hepatitis revealed that he had acute hepatitis E virus (HEV) infection, and the genotype III HEV RNA was detected in the serum sample. This isolate designated HE-JA42 and the sequence of the open reading frame 2 region (412 nucleotides) was registered as the accession number of AB218721 for the DNA databank of Japan, and showed a relatively close identity of approximately 92% with isolates from

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Table 1 Laboratory findings on admission in former hospital

Hematology		Blood chemistry	
Neutrophil	56.6%	D.Bil.	15.7 mg/dL
Lymphocyte	27.6%	AST	1548 IU/L
Monocyte	14.0%	ALT	1483 IU/L
Eosinophil	0.6%	LDH	744 IU/L
Basophil	1.2%	γ-GTP	224 IU/L
White blood cell	4900/μL	T.Bil.	22.2 mg/dL
Red blood cell	436 × 10^4/μL	Al-P	1644 IU/L
Haemoglobin	13.7 g/dL	TBA	197.6 μM/L
Hematocrit	39.8%	T.P.	6.3 g/dL
Platelet	26.2 × 10^4/μL	IgG	1430 mg/dL
		IgA	494 mg/dL
Electrolytes and renal function		IgM	236 mg/dL
Na	141 mEq/L	CRP	1.0 mg/dL
K	4.4 mEq/L		
Cl	107 mEq/L	Blood coagulation	
Urea nitrogen	16.1 mg/dL	PT	145%
Creatinine	0.6 mg/dL	HPT	110%
		Fibrinogen	338 mg/dL
		Antithrombin	156%
		FDP D-dimer	1.1 μg/mL

T.Bil., total bilirubin; D.Bil., direct bilirubin; AST, asparate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; γ-GTP, γ-glutamyltranspeptidase; Al-P, alkaline phosphatase; TBA, total bile acid; T.P., total protein; Ig, Immunoglobulin; CRP, C-reactive protein; PT, prothrombin time; HPT, hepaplastin test (normotest); FDP, fibrin and fibrinogen degradation products.

Table 2 Laboratory findings on admission in Iwate Medical University

Hematology		Blood chemistry		Virus markers	
Neutrophil	46.0%	D.Bil.	26.3 mg/dL	HBsAb	(-)
Lymphocyte	22.0%	AST	427 IU/L	HCVAb	(-)
Monocyte	23.0%	ALT	765 IU/L	EBVCA IgG	(+)
Eosinophil	4.0%	LDH	295 IU/L	EBVCA IgM	(-)
Basophil	1.0%	γ-GTP	295 IU/L	EBNA Ab	(+)
White blood cell	4530/μL	T.Bil.	29.0 mg/dL	HBsAg	(-)
Red blood cell	493 × 10^4/μL	Al-P	1716 IU/L	CMV IgG	(+)
Haemoglobin	15.1 g/dL	T.P.	7.0 g/dL	CMV IgM	(-)
Hematocrit	44.2%	Albumin	3.7 g/dL	HEV IgG	(+)
Platelet	24.6 × 10^4/μL	IgG	1780 mg/dL	HEV IgM	(+)
		IgA	563 mg/dL	HEV RNA	(+)
Electrolytes and renal function		IgM	240 mg/dL	Genotype III	
Na	136 mEq/L	CRP	0.7 mg/dL		
K	4.6 mEq/L			Autoantibodies	
Cl	102 mEq/L	Blood coagulation		ANA	(-)
Urea nitrogen	15.3 mg/dL	PT	114%		
Creatinine	0.8 mg/dL	PT-INR	0.85	Others	
		HPT	115.4%	AFP	2.7 ng/mL
Urinalysis		Fibrinogen	245.8 mg/dL	HGF	0.44 ng/mL
pH	6.0	Antithrombin	112%	4/8 CD	5.25
Sp.G.	1.015	FDP D-dimer	0.5 μg/mL		
Protein	(-)				
Sugar	(-)				

PT-INR, prothrombin time-international normalizaion ratio; HBsAg, hepatitis B surface antigen; HBsAb, hepatitis B surface antibody; EBVCA, Epstein-Barr virus capsid antigen; EBNA, Epstein-Barr virus nuclear antigen; HEV, hepatitis E; ANA, antinuclear antibody; AFP, alpha fetoprotein; HGF, hepatocyte growth factor; 4/8 CD, ratio of clusters of differentiation 4 to 8.

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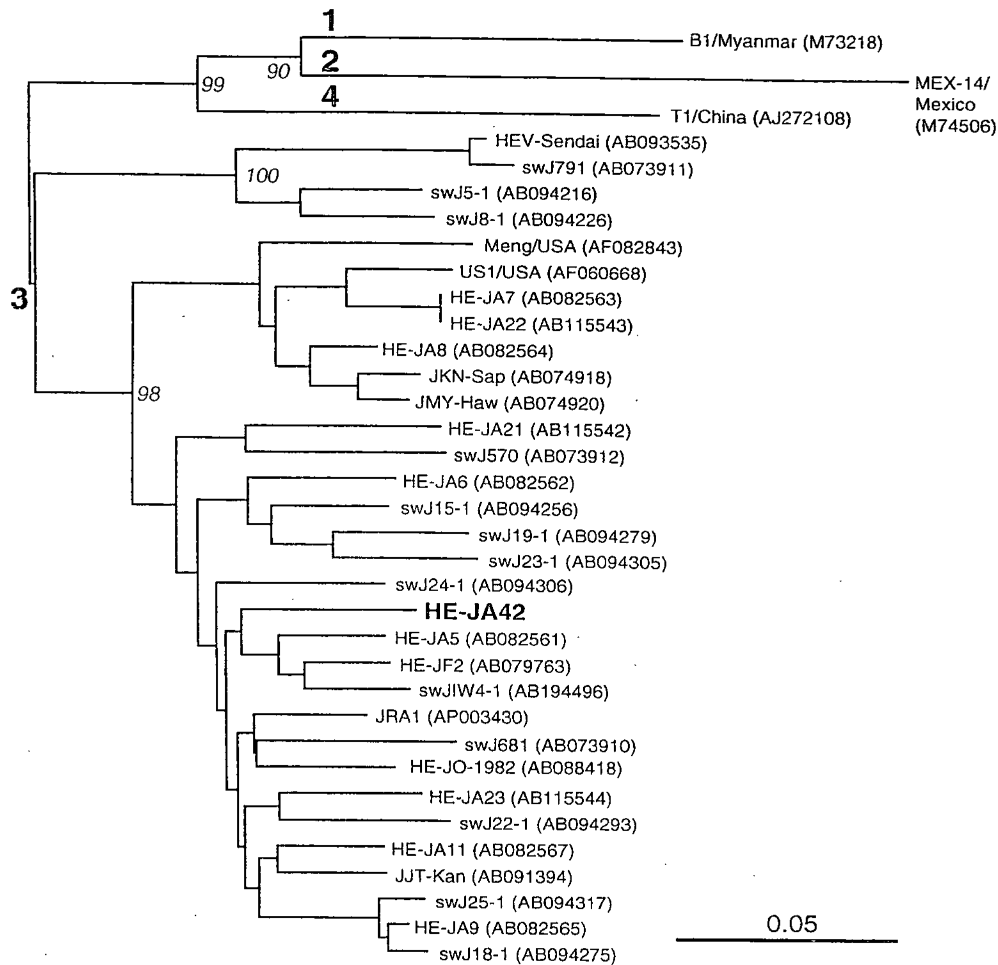

Figure 1 Phylogenetic tree constructed by neighbor-joining method on the basis of partial nucleotide sequence of open reading frame 2 region (301 nucleotides; nt 6037–6337 of the HE-JA10 genome [AB089824]) of reported human and swine genotype III HEV isolates. The HEV isolated from this patient is in bold face (HE-JA 42).

humans (HE-JA5 and HE-JF2) and swine (swJIW4-1) in Iwate prefecture (Fig. 1). Furthermore, a drug-induced lymphocyte stimulation test (DLST) showed a positive result for the drug, Jikinin, which he took for a common cold four weeks before the test. The DLST was carried out as follows: 1×10^6 peripheral blood lymphocytes of the patient per reaction were prepared using Ficoll–Paque, cultured and stimulated by medium with and without the drug solution. Lymphocyte proliferation measured by ^{3}H-incorporation to the DNA was 2.04-fold higher in drug-stimulated lymphocytes than in control. The drug Jikinin is a popular over-the-counter medicine for the common cold, containing some antiphlogistic and analgesic agents as shown in Table 3. Therefore, it was not clear which of these agents was responsible for the hypersensitive response.

Table 3 Active agents and additives included in the drug, Jikinin

Active agents
Dihydrocodeine phosphate
dL-Methylephedrine
Acetaminophen
Chloropheniramine maleate
Anhydrous caffeine
Liquorice extract
Additives
Talc
hydroxypropylcellulose
D-mannitol
Magnesium stearate
Cellulose
Sucrose

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