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Prevalence of Hepatitis E Virus (HEV) Infection in Wild Boars (*Sus scrofa leucomystax*) and Pigs in Gunma Prefecture, Japan

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ABSTRACT. The prevalence of hepatitis E virus (HEV) infection in wild boars and pigs in Gunma Prefecture, Japan, was serologically and genetically examined. The positive detection rates of anti-HEV IgG and HEV RNA in the wild boars were 4.5% (4/89) and 1.1% (1/89), whereas those in the pigs were 74.6% (126/169) and 1.8% (3/169), respectively. The positive rates of anti-HEV IgG and HEV RNA on the 17 pig farms in the present study ranged from 20% to 100%, respectively. One male wild boar approximately 5 years of age was positive for HEV RNA but was negative for anti-HEV IgG. Three pigs from 2 farms were positive for HEV RNA; 2 of these pigs were negative for HEV IgG, and the other was positive. A phylogenetic analysis revealed that all of the HEV ORF1 genes detected in the present study belonged to genotype III. In Gunma Prefecture, HEV is highly prevalent and widespread, and uncooked wild boar and pig meat may have the potential to transmit HEV to humans.

KEY WORDS: hepatitis E virus, Japan, swine, wild boar.

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Hepatitis E virus (HEV), which belongs to the genus *Hepevirus*, is the causative agent of hepatitis E. Hepatitis E infection has been found in many developing countries in Asia, Africa and Latin America, where the disease is an important public health concern [15]. HEV is primarily transmitted by the fecal-oral route such as in waterborne epidemics.

Recent studies have suggested that HEV is divided into 4 genotypes designated as G I, G II, G III, and G IV [17]. The HEV infections in Asia and Africa are mainly caused by G I, and the majority of the GII infection have been reported in Mexico and Nigeria. On the other hand, only a single case of infection with GIII or GIV has been described in the United States, European counties, Argentina, Taiwan and China [17, 21, 22]. In Japan, most imported cases with G I have derived from epidemic areas such as Asia and Africa [2]; however, G III or G IV has also been detected in acute hepatitis patients who have never traveled to HEV epidemic areas [6, 8, 13, 14, 20, 21, 24, 29]. These patients often have a history of consuming uncooked wild boar (*Sus scrofa leucomystax*) and sika deer (*Cervus nippon*) meat and liver [5, 27, 28]. Also, HEV strains belonging to G I, G III or G IV have been detected in Japanese patients with sporadic acute or fulminate hepatitis E [8, 9, 19–22, 24, 31]. In addition, Yazaki *et al.* [31] reported that HEV RNA has been detected in 2% (7/363 packages) of sold pig liver on the market by reverse transcription-polymerase chain reaction (RT-PCR).

In Japan, it has been suggested that the transmission route of HEV remains unclear in approximately 60% of infected patients [1]; zoonotic food-borne transmissions account for 30%, imported infection accounts for 8% and blood transfusion is responsible for 2%. In Gunma Prefecture, Japan, approximately 3,000 wild boars are annually slaughtered for meat [unpublished data], and the number of breeding pigs in the prefecture was approximately 6 million in 2005. According to the Gunma Prefectural Statistics Report (http://www.pref.gunma.jp/cts/PortalServlet?DISPLAY_ID=DIRECT&NEXT_DISPLAY_ID=U000004&CONTENTS_ID=43375), Gunma Prefecture is one of the major pork-producing areas in Japan. However, to the best of our knowledge, there have been no reports on the prevalence of HEV infection in wild boars and pigs in the prefecture to date. Here in, we report the seroprevalence of anti-HEV IgG detected by enzyme-linked immunosorbent assay (ELISA) and HEV RNA by RT-PCR among wild boars and pigs in Gunma Prefecture, Japan.

MATERIALS AND METHODS

Samples: From September 2004 to March 2006, blood samples from 89 wild boars were kindly provided by hunters, and these samples were placed in sterile tubes, stored at approximately 4°C and sent to the laboratory within 12 hr. The ages of the wild boars were estimated by the hunters. From September to December 2004, we collected 169 pig blood samples from 17 pig farms during viscera inspections

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at G slaughterhouse in Gunma Prefecture, with 9 to 10 samples obtained from each farm. All pigs were approximately 6 months old. The blood samples were placed in sterile tubes, stored at approximately 4°C and sent to the laboratory within 3 hr. All blood samples were centrifuged at 1,900 × *g* for 20 min, and the serum was stored at –20°C until analyses.

Serologic analysis: Anti-HEV IgG was measured by ELISA as previously described with some modifications [4]. The antigen used in the ELISA was HEV-like particles composed of a truncated open reading frame 2 (ORF2) protein of genotype I HEV expressed by a recombinant baculovirus in insect cells and was suspended with 0.5 M carbonate buffer (pH 12.5) at a concentration of 1 μg/m*l* [3]. The antigen solution (100 μl) was added to duplicate wells of 96-well microplates (Sumiron ELISA plate type H, Sumitomo Bakelite, Tokyo, Japan). After washing with phosphate buffered saline containing 0.05% of tween-20 (PBST), the wells were coated with 5% skim milk in PBST for 1 hr at room temperature and then incubated with 100 μl of serum samples at a dilution of 1:200 in 1% skim milk in PBST for 1 hr at room temperature. The wells were washed with PBST 3 times, and the bound IgG antibodies were probed with peroxidase-labeled goat anti-swine IgG antibodies (heavy plus light chain; Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.). After washing 3 times with PBST, 100 μl of substrate, for wild boar samples, TMB HRP Microwell substrate, Bio FX Laboratories, MD, USA; for swine samples, 200 μM of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), Sigma, St. Louis, MO, U.S.A.) was added, and the plates were incubated for 10 min (for wild boar samples) or 30 min (for swine samples) at room temperature. Following the incubation period, 100 μl of stop solutions was added to the plates. The density at 450 nm (wild boar samples) or 415 nm (swine samples) was measured using an automatic ELISA reader (Benchmark Plus, BioRad, U.S.A.). A sample was considered positive for anti-HEV IgG when the average of OD value was greater than the cut-off value. To determine the cut-off value of the IgG, each of the 10 samples that had the lowest OD values and were negative in the western blot analysis were used as negative sera. In the present study, ODs of 2.597 and 0.197, which were calculated as three standard deviations above the mean values for the wild boar and swine negative controls, respectively, were used as the tentative cut-off values for each sample.

Extraction of RNA and reverse transcription polymerase chain reaction: Frozen serum samples were thawed at room temperature and then centrifuged at 3,000 × *g* at 4°C for 30 min, and the supernatants were then used for RT-PCR and sequence analysis. Total RNA was extracted from 140 μl of the re-centrifuged serum using a QIAamp Viral RNA Mini kit (Qiagen, MD, U.S.A.). The extracted RNA was then suspended in 60 μl of DNase/RNase-free water and treated with 5 U of DNase I (Takara, Tokyo, Japan). To amplify the 326- nucleotide region from open reading frame 1 (ORF1) by RT-PCR, we used genotype-specific primers as previously described [21]. The amplified DNA fragment was separated by electrophoresis on a 3% agarose gel, and the DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen). The nucleotide sequence was determined using an automated DNA sequencer (ABI PRISM™ 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, U.S.A.) using a Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems). Nucleotide sequences of the partial ORF1 of HEV (positions 124 to 449: 326 bp) were analyzed phylogenetically using CLUSTALW on the DNA database of Japan (DDBJ) homepage (http://hypernig.nig.ac.jp/homology/clustalw-e.shtml) and TreeExplorer (Version 2.12; http://evolgen.biol.metro-u.ac.jp/TE/). Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenic trees were constructed using the neighbor-joining (NJ) method [16]. The reliability of the trees was estimated using 1000 bootstrap replications.

Statistical analysis: The chi-square test with Yates' continuity correction was used to compare the positive detection rates of anti-HEV IgG between male and female wild boars. Differences were considered significant when the p value was less than 0.05.

RESULTS

Prevalence of HEV infection in wild boars in Gunma Prefecture: Anti-HEV IgG was detected in 4 (4.5%) of the 89 wild boars (Table 1). No significant difference was found between for the male (2.9%; 1/35) and female (6.7%; 3/45) wild boars (chi-square test with Yates' continuity correction, p=0.7960). HEV was detected in only 1 wild boar (WBG06–01), giving a 1.1% (1/89) positive rate.

Prevalence of HEV infection in slaughtered pigs in Gunma Prefecture: Anti-HEV IgG was detected in 126 (74.6%) of the 169 slaughtered pigs (Table 2). The positive rates among the individual 17 pig farms varied from 20% to 100%. HEV RNA was detected in 1.8% (3/169) of the pigs, 1 from farm M(PG05–03) and 2 from farm H (PG05–01 and PG02–02), and the positive rates of anti-HEV IgG for these farms were 60% and 89%, respectively.

Information on HEV RNA-positive animals: We detected HEV RNA in one wild boar and three pigs. The wild boar (WBG06–01; male; body weight of about 80 kg) was estimated to be approximately 5 years of age by the hunters and was negative for anti-HEV IgG. Of the 3 pigs, 2 (PG05–01 and PG05–02) were from farm H, and the other (PG05–03) was from farm M. Farms E and L are located in the center of Gunma Prefecture and have no history of contact with wild boars. Of these 3 pigs, 2 (PG05–01 and PG05–03) were negative and 1 (PG05–02) was positive for anti-HEV IgG.

Phylogenetic analysis of the HEV isolates based on the sequences of open reading frame 1: The phylogenetic tree based on the ORF1 gene in HEV detected in Japan and other countries is shown in Fig. 1. The strains were divided into 4 genotypes as described in a previous report [17]. All 4

Table 1. Detection of anti HEV-IgG and HEV RNA in wild boars

Age (months)	Sex	Number of samples	IgG positive samples (%)	HEV RNA detection (%)
< 12	Male	4	0 (0)	0 (0)
	Female	7	0 (0)	0 (0)
13–24	Male	8	0 (0)	0 (0)
	Female	3	0 (0)	0 (0)
25–36	Male	4	1 (25.0)	0 (0)
	Female	10	0 (0)	0 (0)
37–48	Male	6	0 (0)	0 (0)
	Female	7	0 (0)	0 (0)
49–62	Male	1	0 (0)	1[a)](100)
	Female	9	1 (11.1)	0 (0)
>62	Male	5	0 (0)	0 (0)
	Female	6	1 (16.7)	0 (0)
Unknown	Male	7	0 (0)	0 (0)
	Female	3	1 (33.3)	0 (0)
	No record	9	0 (0)	0 (0)
Subtotal	Male	35	1 (2.9)	1 (2.9)
	Female	45	3 (6.7)	0 (0)
	No record	9	0 (0)	0 (0)
Total		89	4 (4.5)	1 (1.1)

a) Sample number: WBG06–01.

Table 2. Detection of anti HEV-IgG and HEV RNA in 17 pig farms

Farm	Number of samples	IgG positive samples (%)	HEV RNA detection (%)
A	10	10 (100)	0 (0)
B	10	10 (100)	0 (0)
C	10	10 (100)	0 (0)
D	10	10 (100)	0 (0)
E	10	10 (100)	0 (0)
F	10	9 (90)	0 (0)
G	10	9 (90)	0 (0)
H	9	8 (88.9)	2[a)](22.2)
I	10	8 (80)	0 (0)
J	10	7 (80)	0 (0)
K	10	7 (70)	0 (0)
L	10	6 (70)	0 (0)
M	10	6 (60)	1[b)](10)
N	10	5 (50)	0 (0)
O	10	5 (50)	0 (0)
P	10	4 (40)	0 (0)
Q	10	2 (20)	0 (0)
Total	169	126 (74.6)	3 (1.8)

a) Sample number: PG05–01 and PG05–02.
b) Sample number: PG05–03.

strains detected in the present study were classified into genotype III, which includes several genotypes of Japanese domestic animals previously reported [8, 11, 12, 20, 23, 28, 31]. The sequences of the 2 pigs (PG05–01 and PG05–02) from farm H were identical (AB362371 and AB362372) but were different from that for farm M by approximately 0.11, while the distances of the wild boar sequence (AB362374) from the sequences of boars from farms H and M were 0.1 and 0.07, respectively.

DISCUSSION

Epidemiological studies have reported that HEV infection is prevalent among wild boars [5, 12, 26, 30] and pigs [10, 25] and have suggested that consumption of the meat and liver of these animals is a risk in terms of HEV infection in Japan [5, 26, 30]. In the present study, the positive rates of anti-HEV IgG and HEV RNA (genotype III) in the wild boars were 4.5% (4/89) and 1.1% (1/89), respectively. The positive detection rates showed no relationship with the age of the animals. Sonoda *et al.* [18] reported that anti-HEV IgG is present in 8.6% (3/35) of wild boars and that HEV RNA genotype III has been detected in a 60-kg male wild boar (2.9%, 1/35) that was negative for anti-HEV IgG (presumed to be approximately 2 years of age). In other study in Japan, Michitaka *et al.* [7] reported a positive rate of anti-HEV IgG in wild boars of 25.5% (100/392), and 3.1% (12/392) of the wild boars in their study were positive for the HEV RNA genotype III. In the present study, although the seroprevalence of HEV infection in the wild boar was considerably lower than in previous reports, some of the animals in Japan are infected with GIII and may potentially serve as a source of infection in humans.

The prevalence of anti-HEV IgG in pigs depends on the age of the animals, and HEV RNA has been detected in 2- to 4-month-old pigs and less commonly in older pigs [10, 14, 23, 25]. Takahashi *et al.* [23, 25] reported detection rates of anti-HEV IgG in 6-month-old pigs that ranged from 73.5% (100/136) to 90.4% (226/250), with no HEV RNA detection from any prefecture examined in Japan to date. Although the positive rates of anti-HEV IgG in the present study were similar to those in previous reports, HEV RNA (genotype III) was detected in 1.8% (3/169) of the pig serum samples, and this suggests that HEV genotype III is highly prevalent and widely distributed in pigs. Thus, it is highly possible that pigs are a source of HEV infection in humans. A nationwide campaign prohibiting consumption of uncooked liver and meat from wild boars and pigs should be implemented to prevent HEV infection in humans.

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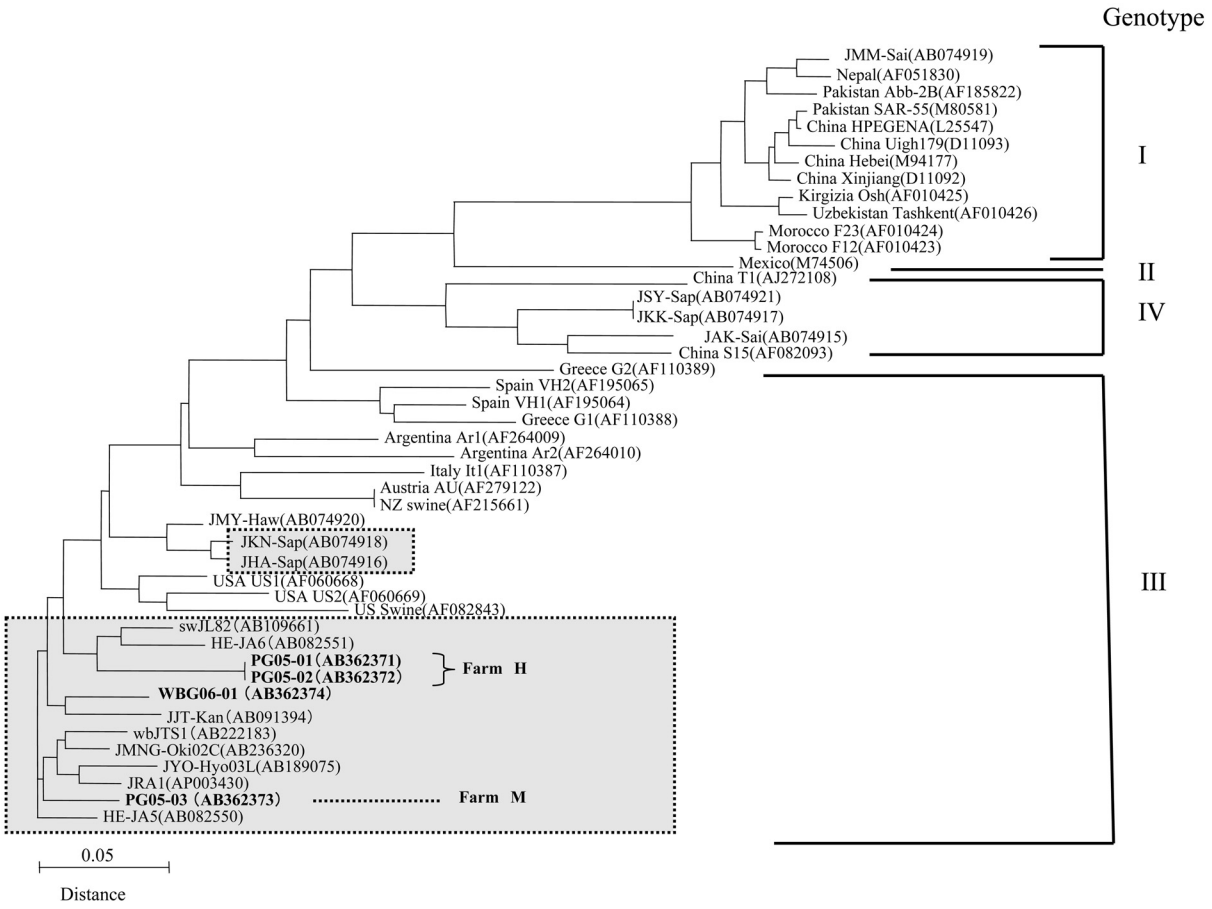

Fig. 1. Phylogenetic trees on the basis of 326 nt of the ORF1 region constructed by the neighbor-joining method [16]. The HEV strains from one wild boar and three pigs from farms are shown in bold type. In addition, genotype III strains reported in previous studies in Japan are indicated by gray boxes. The GenBank accession numbers of the identified strains are enclosed in parentheses.

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