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High Prevalence of Hepatitis E Virus in Wild Boar (*Sus scrofa*) in Yamaguchi Prefecture, Japan

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ABSTRACT: Hepatitis E virus (HEV) causes a food- and water-borne disease in humans, and Japanese wild boar (*Sus scrofa leucomystax*) meat is one of the most important sources of infection in Japan. We tested 113 serum samples from wild boar captured in Shimonoseki City, Yamaguchi Prefecture, Japan from 2010 to 2012. Serum samples were tested by enzyme-linked immunosorbent assay (ELISA) using virus-like particles as antigen and nested reverse-transcription PCR (RT-PCR). Anti-HEV IgG antibodies were detected in 47 of the 113 wild boar serum samples (42%), and HEV RNA was detected in five samples (4%). Sequence analysis showed that the five HEV isolates belonged to genotype 4, forming a cluster with a previous isolate from a human hepatitis E case in this region in 2011. These results indicate that wild boar in this region are infected with potentially pathogenic HEV at a high prevalence.

Key words: Hepatitis E virus, Japan, wild boar, zoonosis.

Hepatitis E virus (HEV) is the causative agent of acute or fulminant hepatitis E in humans. In Asia, Mexico, the Middle East and North Africa, transmission of HEV occurs by the fecal–oral route through contaminated water supplies. In developed countries, HEV has been thought to be imported by travelers from countries where the disease is prevalent. However, there are many reports of patients who were infected while in the United States, Europe, and Japan (Emerson and Purcell 2007). In 2003, people who had consumed uncooked deer (Cervidae) meat were found to be infected with HEV in Japan (Tei et al. 2003). Infection with HEV is now a recognized zoonosis, with deer, domestic pigs (*Sus scrofa domesticus*), and wild boar (*Sus scrofa leucomystax*) acting as reservoirs for human infection in developed countries (Takahashi and Okamoto 2014).

HEV is a nonenveloped, single-stranded positive-sense RNA virus, and it is the only member of the genus *Hepevirus*, family *Hepeviridae* (Emerson and Purcell 2007). There is one HEV serotype, which is genetically divided into four genotypes (Okamoto 2007). Genotypes 1 and 2 are restricted to humans and are often associated with outbreaks in developing countries. Genotypes 3 and 4 cause zoonotic infections in both developing and developed countries (Aggarwal and Jameel 2011). Genotypes 3 and 4 have been isolated from many species, including pigs, wild boars, deer, rats (*Rattus* spp.), rabbits (Leporidae), mongooses (Herpestidae), and horses (*Equus ferus caballus*) (Meng et al. 1997; Nakamura et al. 2006; Saad et al. 2007; Geng et al. 2010; Johne et al. 2010; Cossaboom et al. 2011).

Hepatitis E occurs sporadically in Japan, but the number of infected patients is increasing (Takahashi and Okamoto 2014). The source of the infection is unknown in many cases because the incubation period can be as long as 6 wk. Confirmed cases were recently reported following consumption of pig or wild boar meat (Takahashi and Okamoto 2014). In Yamaguchi Prefecture, in western Honshu, two of the four cases of hepatitis E since 2006 are thought to have resulted from consumption of undercooked wild boar meat. In 2011, one case of hepatitis E was reported in Shimonoseki City, Yamaguchi Prefecture. The patient consumed raw wild boar's liver 1 mo before the onset of symptoms, and this was the presumed source of the virus (Okita et al. 2012). However, the prevalence of HEV in wild boar in the city is unknown. Here, we measured prevalences of anti-HEV IgG

antibody using enzyme-linked immunosorbent assay (ELISA) and HEV RNA using reverse-transcription PCR (RT-PCR) in samples from wild boar in Shimonoseki City, Yamaguchi Prefecture, Japan.

We collected 113 serum samples from wild boar in mountainous regions of Shimonoseki City, Yamaguchi Prefecture, Japan (34°18′6″N, 130°58′52″E). Most animals were hunted with government permission during the winter season, and blood was collected from the heart using a 21G needle and 50 mL syringe (Nipro, Osaka, Japan). We collected 71 serum samples from wild boar captured in the mountainous region in Wakayama Prefecture (33°45′36″N, 135°23′11″E) between 2007 and 2010. After centrifugation, sera were collected and kept at −20 C until use. Liver samples were also collected from most wild boar, carried to the laboratory at 4 C, and then stored at −80 C until use.

Anti-HEV IgG was detected in sera by ELISA, using virus-like particles (VLPs) as the coating antigen (Yamashita et al. 2009). The VLPs were diluted in 0.1 M carbonate buffer (pH 9.6) to 1 μg/mL, and 100 μL per well was added to 96-well microplates (Nunc, Roskilde, Denmark). The wells were blocked with 0.1% bovine serum albumin in phosphate-buffered saline (PBS) for 1 hr at 37 C. After three washes with PBS containing 0.05% tween-20 (PBS-T), wild boar serum at a dilution of 1:100 in PBS-T containing 10% fetal calf serum (FCS) was added for 1 hr at 37 C. After the plate was washed with PBS-T three times, horseradish peroxidase–conjugated rabbit anti-swine IgG (MP Biomedicals, Carlsbad, California, USA), diluted 1:1000 in PBS-T containing 10% FCS was added for 1 hr at 37 C. After a further three washes with PBS-T, horseradish peroxidase substrate (BioRad, Hercules, California, USA) was added to each well for 30 min at room temperature. After stopping the reaction with 2% oxalic acid, the absorbance at 415 nm was measured using a spectrophotometer (BioRad).

We used RT-PCR to detect HEV RNA in sera. Viral RNA was extracted from 140 μL of each serum sample using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany). To amplify the partial gene of ORF2, RT-PCR was carried out with primers HEV-F1 (5′-TAY CGH AAY CAA GGH TGG CG-3′) and HEV-R2 (5′-TGY TGG TTR TCR TAR TCC TG-3′) using a One Step RT-PCR kit (Qiagen). Nested PCR was performed with the internal primers HEV-F2 (5′-GGB GTB GCN GAG GAG GAG GC-3′) and HEV-R1 (5′-CGA CGA AAT YAA TTC TGT CG-3′) using a KOD-Plus-Ver.2 (Toyobo, Osaka, Japan) (Yamamoto et al. 2008). The amplified PCR products were extracted from the gel using a QIAEX II Gel Extraction Kit (Qiagen). The nucleotide sequence was determined using BigDye Terminator v.3.1 technology (Applied Biosystems, Foster City, California, USA) with primers HEV-F2 and HEV-R1. The five nucleotide sequences were deposited in the DNA Data Bank of Japan (DDBJ) as accession numbers AB746334, AB746335, AB746336, AB746337, and AB746338. Furthermore, the junction between ORF1 and ORF2 was amplified by RT-PCR from liver samples of three HEV-RNA–positive wild boar (Shimonoseki-WB72, -WB97, and -WB99) using a different set of primers; HE-008 (5′-GGG GTT GGT TGG ATG AAT ATA GGG GA-3′) and HE-015 (5′-TGG AAG AAR CAY TCY GGT GAG CC-3′) (Takahashi et al. 2002). Japanese HEV isolates have been detected successfully using this method.

We assayed 113 serum samples from wild boar in Shimonoseki City and 71 from boars in Wakayama Prefecture for anti-HEV IgG by ELISA. All wild boar samples in Wakayama Prefecture were negative for HEV (Fig. 1). Mean and standard deviation (SD) of the optical density values obtained from the sera of wild boar from Wakayama Prefecture were 0.099 and 0.075, respectively

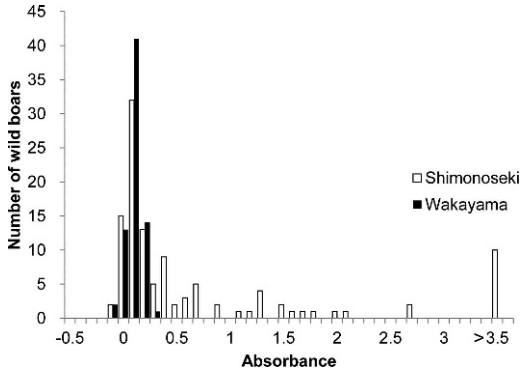

FIGURE 1. Distribution optical density values for enzyme-linked immunosorbent assay to measure prevalence of hepatitis E virus in sera from 113 wild boars (*Sus scrofa*) in Shimonoseki City and 71 wild boars in Wakayama Prefecture Japan.

(Fig. 1). The cutoff value was determined as 0.324 (mean±3×SD). When the ELISA results were analyzed using a cutoff value of 0.324, none of 71 wild boars from Wakayama Prefecture and 47 of 113 (42%) from Shimonoseki City, were positive for HEV. There was no significant difference in HEV antibody prevalence by sex or body weight ($P>0.05$; Table 1).

HEV-RNA was detected in sera from five of 112 wild boar (4%) captured in Shimonoseki City (Shimonoseki-WB52, -WB72, -WB74, -WB97, and -WB99). All five HEV-RNA–positive samples were from boars captured in Kanda or Imade, in which the antibody prevalences of HEV (68% and 56%, respectively) were higher than those in boars from the other sites. Sequence analysis of the amplified products (338 base pairs) classified all isolates detected in Shimonoseki City as genotype 4. Nucleotide and amino acid sequences of these isolates showed identities of 95.3–100% and 99.2–100%, respectively. These strains had high homology with a Chinese strain, CCC220 (88.7–89.4% in the nucleotide sequence and 89.9–90.5% in the amino acid sequence), but had less than 83.2% identity with the other Japanese strains.

The junction between ORF1 and ORF2 was amplified by RT-PCR from the liver samples of three HEV-RNA–positive wild boars (Shimonoseki-WB 72, -WB97, and -WB99) because some Japanese HEV isolates have previously been detected using this method. We detected HEV RNA in liver samples by single RT-PCR, indicating that HEV might be present at higher levels in liver than sera. These three nucleotide sequences were deposited to DDBJ as accession numbers AB746339, AB746340, and AB746341. The nucleotide sequences confirmed that these isolates were also classified as genotype 4. Nucleotide sequence identities between these isolates were 97.4–100% and there was also high homology with CCC220 (91.7–91.8%) (data not shown).

A human hepatitis E case was reported in Shimonoseki City in March 2011 in which the patient had consumed undercooked wild boar liver 1 mo before hospitalization (Okita et al. 2012). Recently, the complete genome sequence from this case, JTF-Yamagu11, was reported (DDBJ Accession AB698654). The nucleotide sequence of the region amplified with HEV-F1 and HEV-R2 from this strain had high homology to all of the

TABLE 1. Prevalence of hepatitis E virus infection in wild boar in Shimonoseki City, Japan, as detected by enzyme-linked immunosorbent assay (ELISA) or reverse-transcription PCR (RT-PCR). Number of positive animals/number examined (percentage positive).

	Sex		Body weight (kg)			
	Male	Female	<20	20–50	>50	Total
ELISA	16/44 (36)	31/69 (45)	6/21 (29)	19/45 (42)	22/47 (47)	47/113 (42)
RT-PCR	2/44 (5)	3/68 (4)	2/21 (10)	1/44 (2)	2/47 (4)	5/112 (4)

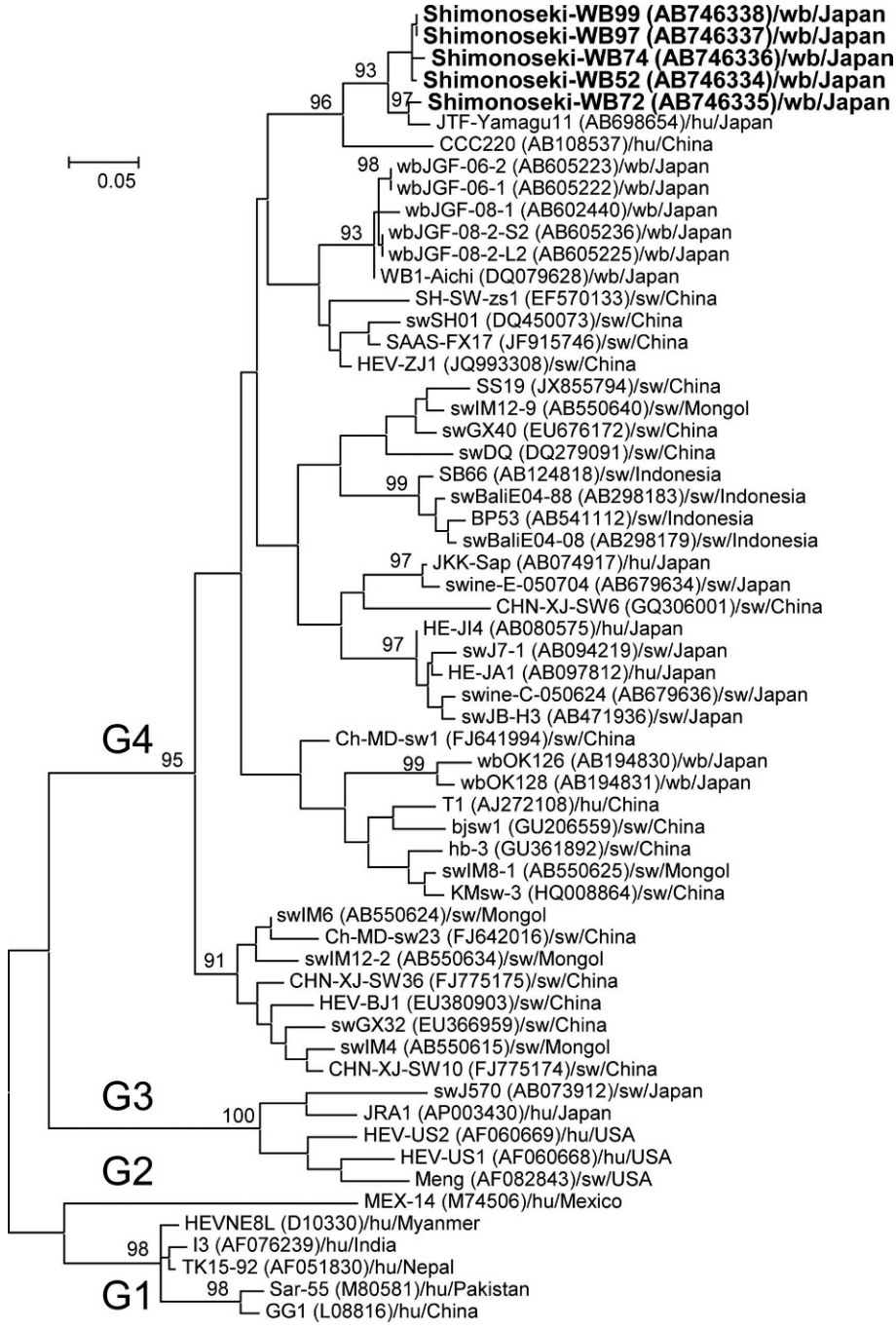

FIGURE 2. Maximum likelihood phylogenetic tree constructed using MEGA 5.05. Hepatitis E genes were amplified from serum samples of Japanese wild boars (*Sus scrofa*) by nested reverse-transcription PCR, and the nucleotide sequences (338 base pairs) were obtained by sequencing using primers HEV-F2 and HEV-R1. The number in the nodes indicates the bootstrap percentage (of 1,000 replicates). Our analyzed samples are indicated in bold. wb = wild boar, sw = domestic pig, hu = human.

wild boar isolates from Shimonoseki City (95.3–97.6% in the nucleotide sequence, and 99.2–100% in the amino acid sequence) (Fig. 2). Furthermore, the nucleotide sequence of the region amplified with primers HE-008 and HE-015 indicated that this human isolate was also very similar to HEV isolated from wild boar from Shimonoseki City (97.0–97.9% nucleotide sequence homology) (data not shown). This confirmed that the patient who developed hepatitis E after consuming uncooked wild boar liver in Shimonoseki City was infected with a strain closely related to that isolated from wild boar, suggesting that HEV in wild boar in Shimonoseki City might be pathogenic for humans.

The prevalence of antibody to HEV in wild boar in Shimonoseki City (47%) was higher than the 8% average level reported in Japan (Sato et al. 2011). These researchers used analytical methods similar to those used in our study, but their cutoff value was calculated as mean± 6×SD using swine serum samples as negative controls. When our results were analyzed using a cutoff value of 0.55 (mean±6×SD), HEV antibody prevalence in wild boar in Shimonoseki City (31%) was still higher than the average level reported in Japan. These results confirm that HEV is endemic among wild boar in Shimonoseki City. Although the prevalence of potentially pathogenic HEV in wild boar in Shimonoseki City was high, the etiology of HEV in wild animals, including wild boar and deer, is still unclear; further studies are needed.

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