

## The high prevalence of hepatitis E virus infection in wild boars in Ibaraki Prefecture, Japan

Takumi MOTOYA<sup>1,2</sup>\*, Noriko NAGATA<sup>1</sup>, Harumi KOMORI<sup>1</sup>, Ikuko DOI<sup>1</sup>, Miho KUROSAWA<sup>1</sup>, Toshimasa KETA<sup>1</sup>, Nobuya SASAKI<sup>2</sup> and Koji ISHII<sup>3</sup>

<sup>1</sup>Ibaraki Prefectural Institute of Public Health, 993-2 Kasahara-cho, Mito, Ibaraki 310-0852, Japan

<sup>2</sup>Laboratory of Laboratory Animal Science and Medicine, Faculty of Veterinary Medicine, Kitasato University, Higashi 23-35-1, Towada, Aomori 034-8628, Japan

<sup>3</sup>Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan

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**ABSTRACT.** Hepatitis E virus (HEV) is known as a causative agent of zoonosis and food poisoning. Pigs and some species of wild animals, including wild boar, are known to be a reservoir of HEV. In this study, we investigated the situation regarding HEV infection in wild boars in Ibaraki Prefecture, Japan. Serum, liver and feces samples from 68 animals were collected, and the presence or absence of HEV genomic RNA and HEV antibodies were analyzed. The viral genome was detected in samples from 7 (10.3%) animals, with all HEVs classified as genotype 3, subtype 3b. HEV antibodies were detected in samples from 28 (41%) animals. This report demonstrates for the first time the high prevalence of HEV infection in wild boars in Ibaraki Prefecture.

**KEY WORDS:** ELISA, genotype, hepatitis E virus, phylogenetic analysis, wild boar

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Hepatitis E virus (HEV) is a small, non-enveloped virus with a single stranded, positive sense RNA genome that is approximately 7.2 kb in size [4, 13]. HEV is transmitted mainly via a fecal–oral route and is the causative agent of hepatitis E, which is an important public health disease in many developing countries [21]. HEV infection in developing countries is mostly a waterborne disease associated with large epidemics due to the contamination of water and water supplies as well as to poor sanitary conditions [2, 14]. In contrast, in industrialized countries, including many European countries, the U.S.A. and Japan, acute hepatitis E occurs sporadically, and the contamination pathways are still not fully understood [14]. In Japan, four cases of hepatitis E have been linked directly to the consumption of raw deer meat [17], and several cases of acute hepatitis E have been epidemiologically linked to the consumption of undercooked pork liver or wild boar meat [8, 10, 20]. These cases provide convincing evidence of zoonotic food-borne HEV transmission.

At least four HEV genotypes are known to exist among infections in humans [9]. Most stocks separated in Asia, including Myanmar, are genotype 1 (G1), while the Mexican stocks, which caused a widespread infection in Mexico in the 1990s, are genotype 2 (G2). Genotypes 3 (G3) and 4 (G4) were separated from a scattering of cases in the U.S.A. and China late in the 1990s, respectively. Unlike HEV G1

and G2, HEV G3 and G4 infect not only humans but also animals, such as the wild boar and pig [11, 18]. HEV strains obtained from humans and animals in Japan generally belong to G3 or G4, and HEV G3 strains indigenous to Japan have been provisionally classified into three sub-genotypes: 3b (3jp), 3a (3us) and 3e (3sp), where “jp” stands for Japan type, “us” for US type and “sp” for Spanish (European) type [12]. In this study, we investigated the HEV infection status in wild boars in three areas of Ibaraki Prefecture, Japan. Serum, liver and feces samples were extracted from a total of 68 wild boars culled as part of a noxious animal extermination program in three areas of Ibaraki Prefecture from December 2013 to March 2014.

Blood samples were centrifuged at 1,500 g for 10 min to separate the serum. Liver and fecal samples were suspended in phosphate-buffered saline at 1:10 (weight/volume) and then homogenized. The supernatant fluids were then clarified by centrifugation at 10,000 g for 10 min. Virus nucleic acid was extracted using a QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany), treated with 10 U of DNase I (Takara, Kyoto, Japan) and reverse transcribed using PrimeScript RT Master Mix (Takara). HEV genotypes were classified based on ORF2 sequences as the ORF2 region is well conserved across all four genotypes [12]. The resulting cDNA samples were amplified by real-time PCR using the gene-specific primers [7]. For genotyping for HEV classification, a 378-bp segment of the capsid domain in ORF2 was amplified using a PrimeScript II High Fidelity One Step RT-PCR kit (Takara) and subjected to sequence determination using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) as described previously [16]. A phylogenetic tree was constructed according to the neighbor-joining method with 1,000 bootstrap analyses using MEGA6 software (<http://www.megasoftware.net>).

\*CORRESPONDENCE TO: MOTOYA, T., Ibaraki Prefectural Institute of Public Health, 993-2 Kasaharacho, Mito, Ibaraki 310-0852, Japan. e-mail: t.motoya@pref.ibaraki.lg.jp

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Moreover, sera were used for antibody test to detect IgM and IgG [19]. The OD cutoff values were set at 0.25 and 0.15 for IgG and IgM, respectively.

Ibaraki Prefecture is located in the southeast of Japan and is partly mountainous in nature (Fig. 1). Table 1 shows the results of real-time PCR and conventional PCR and ELISA tests. The positive rate for HEV RNA in area A was 23.53% (4/17), and the antibody-positive rate was 58.82% (10/17), which were the highest among the three areas. The positive rate for HEV RNA in area B was 0% (0/23), and the antibody-positive rate was 8.69% (2/23). The positive rate for HEV RNA in area C was 10.71% (3/28), and the antibody-positive rate was 57.14% (16/28).

HEV RNA could be amplified from 7 of the 68 samples tested (10.3%). PCR products obtained from the HEV-positive wild boars were subjected to direct sequencing and genotyping. We found 35 polymorphic nucleotides in ORF2 (Table 2). Almost all of polymorphisms were silent mutations. Interestingly, intrahost sequence diversity was found in two boars (A-10 and C-2). In particular, missense substitutions (G6107A and S325N) were found in a serum sample from C-2. Several studies have suggested the existence of intrahost quasi-species in both humans and pigs [3, 5]. Potential PCR bias due to low template concentrations might also play a part in the heterogeneous target population. Although this study did not determine coinfection or quasi-species, further studies should take these phenomena into account.

Phylogenetic tree analysis revealed that all of the HEV strains detected in this study could be classified as G3, subgenotype3b (3jp) and were grouped into two clusters; one group consisting of isolates from area A and the second group consisting of those from area C (Fig. 2). The samples from the wild boars taken from the same mountain were classified into the same cluster. It is suggesting that different HEV strains were circulating in the two separate areas (A and C).

The PCR and IgG ELISA positive rates for areas A and C were higher than those for area B. Area A was located in the center, B in the north and C in the south of Ibaraki Prefecture (Fig. 1). The distance between A and B was approximately 15 km, that between A and C was over 40 km, and B and C were even more separated. It has been reported that the range of a wild boar is about 10 km in general, with some individuals ranging over as much as 50 km [15]. However, we speculate that there would be no traffic between the wild boars in each area as two rivers divided the three areas. Thus, genetic similarity and infection rate depended on distinct geographic area.

HEV replicates in liver cells, and virions are secreted into the intestinal lumen via the bile duct and subsequently excreted within the feces. Thus, we tried to detect HEV RNA from the liver and feces. In the case of humans, IgM anti-HEV antibodies can be detected during the first few months after HEV infection, while anti-HEV IgG usually persists for many years after infection. The presence of HEV RNA indicates current infection, whether acute or chronic [1]. In this study, the virus was detected from four individuals in

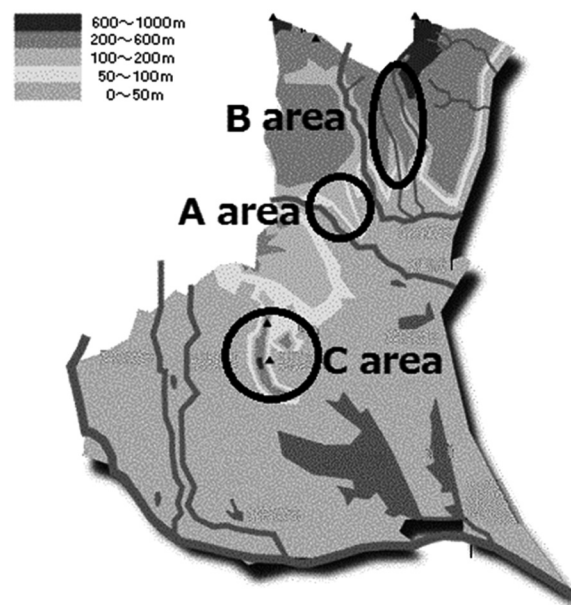


Fig. 1. Map of Prefecture Ibaraki, Japan. Showing the locations in which the wild boars were captured. (Areas A, B and C)

area A and three individuals in area C, and all had higher IgG antibody values and lower IgM antibody values. In contrast, three boars (C-3, C-8 and C-11) showed higher (>0.3) anti-HEV IgM antibodies. These phenomena suggested that IgM was induced by infection with low amounts of virus and that the virus was soon lost. The amount of virus in the body depends on the level of viral exposure and the timing of capture. HEV was also detected from the individuals with high anti-HEV IgG titers, we speculated re-infection.

Although Ibaraki Prefecture has been working on measures for the extermination of wild boars, crop damage still occurs and some expansion of their habitat and area of damage has been observed [6]. It is expected that HEV is present in the liver, feces and serum of wild boars in the HEV-contaminated areas, and the results of this study demonstrate that careful handling of animals is required. Currently, each municipality in Ibaraki Prefecture entrusts the extermination of noxious animals to hunting companies. Thus, it is highly possible that wild boars are a source of HEV infection in humans. A nationwide campaign prohibiting the consumption of uncooked liver and meat containing blood from wild boars should be implemented to prevent HEV infection in humans. It is very important not only for hunting club members but also general residents to thoroughly understand the precautions to be taken when handling of wild animals and their products in order to avoid viral infection.

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Table 1. Detection of HEV RNA and antibodies against HEV in the results of real-time PCR, conventional PCR and antibody tests

Area	No.	qPCR			PCR			ELISA	
		Serum	Liver	Feces	Serum	Liver	Feces	IgG	IgM
A	1	-	-	-	-	-	-	+ (2.752)	-
	3	+	+	-	+	+	-	+ (2.785)	-
	4	+	+	+	+	+	+	+ (3.642)	-
	7	+	+	NT	+	+	NT	+ (3.596)	-
	10	-	+	+	+	-	+	+ (3.644)	-
	11	-	-	-	-	-	-	+ (2.777)	-
	12	-	-	-	-	-	-	+ (0.25)	-
	14	-	-	-	-	-	-	+ (2.902)	-
	16	-	-	-	-	-	-	+ (0.26)	-
	17	-	-	-	-	-	-	+ (0.847)	-
Other 7 boars		-	-	-	-	-	-	-	-
B	2	-	-	-	-	-	-	+ (0.395)	+ (0.203)
	12	-	-	NT	-	-	NT	-	-
	22	-	-	-	-	-	-	+ (0.581)	-
	Other 20 boars		-	-	-	-	-	-	-
C	1	-	-	-	-	-	-	+ (1.377)	-
	2	+	+	+	+	+	+	+ (3.163)	-
	3	-	-	-	-	-	-	+ (1.007)	+ (0.438)
	4	-	-	-	-	-	-	+ (0.26)	-
	5	-	-	-	-	-	-	+ (2.29)	-
	6	-	+	-	+	-	-	+ (3.636)	+ (0.191)
	8	-	-	-	-	-	-	+ (0.913)	+ (0.523)
	10	-	-	-	-	-	-	+ (3.475)	-
	11	-	-	-	-	-	-	+ (1.174)	+ (0.319)
	12	-	-	-	-	-	-	+ (0.35)	+ (0.203)
	13	-	-	-	-	-	-	+ (1.21)	-
	18	-	-	-	-	-	-	+ (3.608)	+ (0.146)
	19	-	+	-	-	-	+	+ (1.597)	-
	22	-	-	-	-	-	-	+ (1.866)	-
24	-	-	-	-	-	-	+ (0.4)	-	
28	-	-	-	-	-	-	+ (0.499)	+ (0.225)	
Other 12 boars		-	-	-	-	-	-	-	-

Serum, liver and feces samples were used for the genetic tests and serum samples for the antibody test. (+) represents; detected, (-); not detected and NT; not tested as samples were not available. Number in anti-body test results indicated ELISA titers. Results obtained from the 3 areas in which the wild boars were captured are shown separately, A, B and C.

Table 2. Comparison of the nucleotide sequences of HEV detection in this study

Individual	Sample	Nucleotide position (bp)																																		
		5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6										
A-3	Serum	A	C	C	C	T	A	C	C	C	C	C	G	C	A	C	T	A	C	G	C	A	T	C	T	G	A	A	G	G	G	T	T	T	T	
	Liver	A	C	C	C	T	A	C	C	C	C	C	G	C	A	C	T	A	C	G	C	A	T	C	T	G	A	A	G	G	G	T	T	T	T	
A-4	Serum	A	C	C	C	T	A	C	C	C	C	C	G	C	A	C	T	A	C	G	C	A	T	C	T	G	A	A	G	G	G	T	T	T	T	
	Liver	A	C	C	C	T	A	C	C	C	C	C	G	C	A	C	T	A	C	G	C	A	T	C	T	G	A	A	G	G	G	T	T	T	T	
	Feces	A	C	C	C	T	A	C	C	C	C	C	G	C	A	C	T	A	C	G	C	A	T	C	T	G	A	A	G	G	G	T	T	T	T	
A-7	Serum	A	C	C	C	T	A	C	C	C	C	C	G	C	A	C	T	A	C	G	C	A	T	C	T	G	A	A	G	G	G	T	T	T	T	
	Liver	A	C	C	C	T	A	C	C	C	C	C	G	C	A	C	T	A	C	G	C	A	T	C	T	G	A	A	G	G	G	T	T	T	T	
A-10	Serum	G	C	C	C	T	A	C	C	C	T	C	G	C	A	C	T	A	C	G	C	A	T	C	T	G	A	A	G	G	G	T	T	T	T	
	Feces	G	T	C	C	T	A	C	C	C	T	C	G	C	A	C	T	A	C	G	C	A	T	C	T	G	A	A	G	G	G	T	T	T	C	
C-2	Serum	A	T	T	T	C	C	T	T	T	T	A	T	T	T	C	G	T	G	T	G	T	G	C	C	C	T	G	T	A	G	A	C	T	C	T
	Liver	A	T	T	T	C	C	T	T	T	T	A	T	T	T	C	G	T	G	T	G	T	G	C	C	C	T	G	T	A	G	A	C	C	C	T
	Feces	A	T	T	T	C	C	T	T	T	T	A	T	T	T	C	G	T	G	T	G	T	G	C	C	C	T	G	T	A	G	A	C	C	C	T
C-6	Serum	A	T	T	T	C	C	T	T	T	T	A	T	T	T	C	G	T	G	T	G	T	G	C	C	C	T	G	T	A	G	A	C	C	C	T
C-19	Feces	A	T	T	T	C	C	T	T	T	T	G	T	T	T	C	G	T	G	T	G	T	G	C	T	C	T	G	T	A	A	A	C	T	C	T

Sequence variations were colored gray. Sequence data were deposited in GenBank under accession number D10330.

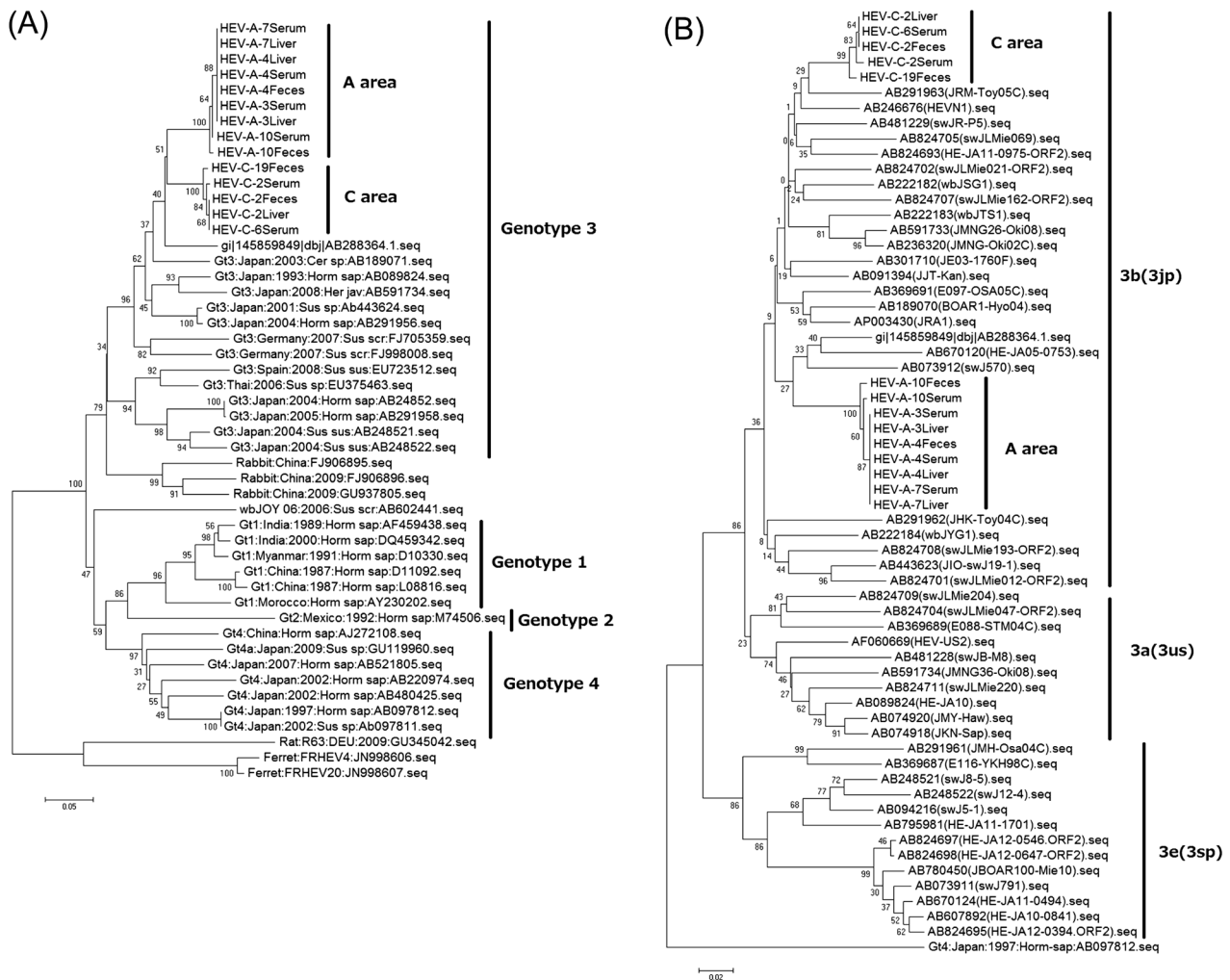


Fig. 2. Phylogenetic tree constructed on the basis of partial sequences of the HEV capsid gene. (A) Genotyping of detected HEV strains. The tree was constructed with reference sequences of HEV genotypes using the neighbor-joining method. The scale bar represents 0.05 nucleotide substitutions per position. (B) Subtyping of detected HEV strains. The tree was constructed with reference sequences of HEV three subgenotypes of G3 with reference sequences of G3 and an outgroup isolate of G4 using the neighbor-joining method. Indigenous Japanese G3 isolates are divided into three subgenotypes: 3a, 3b and 3c. The scale bar represents 0.02 nucleotide substitutions per position.

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