Biotechnology) or anti-ORF3 mAb, respectively. These proteins were visualized by chemiluminescence using the Clean-Blot IP Detection Reagent (HRP) (Thermo Scientific).

**Immunofluorescence assay.** PLC/PRF/5 cells in a four-well chamber slide (Nunc) were transfected with 0.5 μg each of pBj-Myc-Tsg101 and pCI-HEVORF3/wt or pCI-HEVORF3/mutPLAP/LSAL using the TransIT-LT1 reagent (Mirus Bio) according to the manufacturer's instructions. The pBj-Myc and pCI vectors were used as negative controls. For staining of the transfected cells, anti-Myc (9E10) and anti-ORF3 (TA0536) mAbs were used to label cells by using the Zenon Alexa Fluor-488 mouse $IgG_1$ and Zenon Alexa Fluor-594 mouse $IgG_{2a}$ labelling kits (Molecular Probes), respectively, according to the manufacturer's instructions. For staining of CD63 in the HEV-infected cells, anti-CD63 polyclonal antibody (H-193; Santa Cruz Biotechnology) labelled by using the Zenon Alexa Fluor-488 rabbit IgG labelling kit (Molecular Probes) was used 48 h post-transfection, cells were fixed in 4 % paraformaldehyde at room temperature for 15 min and permeabilized in PBS containing 0.2 % (v/v) Triton X-100 at room temperature for 10 min. Non-specific binding was blocked with 1 % (w/v) BSA in PBS at room temperature for 30 min. Zenon labelling complexes were diluted to a final dilution of 1 : 50 for both primary antibodies, and were applied to the cells at room temperature for 2 h. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Roche). The stained cells were viewed under a FV1000 confocal laser microscope (Olympus). In the quantification of co-localized cells (mean ± SE), at least 20 cells were used for calculations in each of two independent experiments.

**RNA interference.** The following siRNAs were obtained from Dharmacon and used in the present study: human Tsg101 (siGENOME SMARTpool M-003549-01-0005) and control siRNA (siGENOME Non-Targeting siRNA Pool #1 D-001206-13-05). The PLC/PRF/5 cells were seeded at a density of $6.0 \times 10^4$ cells per well of a 24-well plate (BD Falcon) in antibiotic-free growth medium. The cells were transfected with 5 nM (final concentration) siRNA in Opti-MEM (Gibco/Invitrogen) using DharmaFECT 1 (Dharmacon) according to the manufacturer's instructions, 2 days before and 4 days after virus inoculation.

**Plasmid transfection.** To analyse the effects of overexpression of Vps4 DN mutants, PLC/PRF/5 cells ($6.0 \times 10^4$ cells per well) in 24-well plates were transfected with 0.5 μg of the expression plasmids for wild-type Vps4 (Vps4A or Vps4B) or their DN mutants (Vps4AEQ or Vps4BEQ) using TransIT-LT1 (Mirus Bio) according to the manufacturer's instructions, 2 days before and 2 and 6 days after virus inoculation. The empty vector, pCDNFL, which was constructed from pcDNA3.1 (Invitrogen) to express a protein containing a FLAG tag at the N terminus, was used as a control.

**Virus inoculation.** Monolayers of PLC/PRF/5 cells in 24-well plates, which were pretreated with siRNA against Tsg101, or the DN forms of Vps4A and Vps4B as described above, were inoculated with $2.0 \times 10^5$ copies of HEV progenies in the culture supernatant of pJE03-1760F/wt RNA-transfected cells. After incubation at room temperature for 1 h, the cells transfected with siRNA or expression plasmids for Vps4A, Vps4B and their derivatives were washed with PBS, 0.5 ml of antibiotic-free or complete growth medium, respectively, was added to each well, and the cells were incubated at 37 °C. Every other day, half of the culture medium (0.25 ml) of siRNA- or Vps4 plasmid-transfected cells was replaced with antibiotic-free or complete growth medium, respectively. The collected culture medium was centrifuged at 1300 *g* at room temperature for 2 min and the supernatant was stored at −80 °C until use.

**Western blotting analysis.** The transfected cells were lysed in lysis buffer [50 mM Tris/HCl (pH 8.0), 1 % NP-40, 150 mM NaCl and protease inhibitor cocktail (Sigma)], and proteins in the cell lysates were separated by SDS-PAGE. The proteins were blotted onto PVDF (0.45 μm; Millipore), immunodetected with an anti-HEV ORF3 (TA0536), anti-Myc (9E10), anti-Tsg101 (C-2), anti-Flag (FLAG M2; Sigma) or anti-β-actin (Sigma-Aldrich) mAb, and then visualized by chemiluminescence, as described previously (Yamada *et al.*, 2009b).

**Quantification of HEV RNA.** RNA extraction from culture supernatants was performed using the TRIzol-LS Reagent (Invitrogen). Intracellular RNA was extracted from cultured cells using the TRIzol Reagent (Invitrogen). The quantification of HEV RNA was performed by real-time reverse transcription (RT)-PCR using a LightCycler apparatus (Roche), with a QuantiTect Probe RT-PCR kit (Qiagen) and two sets of primers and a probe targeting the ORF2 and ORF3 overlapping region or the ORF1 region, as described previously (Takahashi *et al.*, 2008a; Yamada *et al.*, 2009a). Unless otherwise stated, the quantification of HEV RNA was performed using the real-time RT-PCR method with the primers and probe derived from the ORF2/ORF3 overlapping region.

## ACKNOWLEDGEMENTS

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and from the Ministry of Health, Labour, and Welfare of Japan.

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Arch Virol (2011) 156:1345–1358
DOI 10.1007/s00705-011-0988-x

ORIGINAL ARTICLE

# A nationwide survey of hepatitis E virus (HEV) infection in wild boars in Japan: identification of boar HEV strains of genotypes 3 and 4 and unrecognized genotypes

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Received: 6 January 2011 / Accepted: 21 March 2011 / Published online: 8 April 2011
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**Abstract** To investigate the nationwide prevalence of hepatitis E virus (HEV) infection and to characterize HEV genomes among Japanese wild boars (*Sus scrofa leucomystax*), 578 boars captured in 25 prefectures from 2003 to 2010 were studied. Anti-HEV IgG was detected in 8.1%, and HEV RNA in 3.3% of boars. Among the 19 boar HEV isolates obtained from infected boars, 14 isolates (74%) were classified as genotype 3, 4 isolates (21%) as genotype 4, and the remaining isolate (wbJOY_06) was distantly related to all known HEV isolates of genotypes 1-4, differing by 18.4-25.0% and 18.0-24.3% within the 412-nucleotide sequence of ORF1 and ORF2, respectively. A genotype 4 boar HEV isolate (wbJGF_08-1) obtained herein shared 98.6% identity over the entire genome with a human HEV isolate obtained from a patient who developed acute hepatitis after consuming undercooked wild boar meat, suggesting that wild boars are also reservoirs for genotype 4 HEV in humans.

The nucleotide sequences of boar or human HEV isolates reported herein have been assigned DDBJ/EMBL/GenBank accession nos. AB602439-AB602440 (full-length genome), AB605180-AB605209 (ORF1, 412 nt), and AB605210-AB605239 (ORF2, 412 nt).

**Electronic supplementary material** The online version of this article (doi:10.1007/s00705-011-0988-x) contains supplementary material, which is available to authorized users.

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## Introduction

The hepatitis E virus (HEV), the causative agent of acute or fulminant hepatitis E in humans, is an important public health concern in many developing countries in Asia, Africa, and Latin America, where sanitation conditions are suboptimal [38]. In contrast, sporadic cases of hepatitis E that are not related to travel to endemic areas, most likely of zoonotic origin, have been increasingly recognized in many industrialized countries including the United States, European countries, and Japan [1, 10, 17, 32, 36].

HEV is a single-stranded, positive-sense RNA virus without an envelope, and it belongs to the genus *Hepevirus* of the family *Hepeviridae* [6]. The genome of HEV is approximately 7.2 kb in size and contains a short 5' untranslated region (5'UTR), three open reading frames (ORFs: ORF1, ORF2 and ORF3) and a short 3' UTR terminated by a poly (A) tract [49]. ORF1 encodes nonstructural proteins involved in replication, ORF2 codes for a capsid protein of 660 amino acids (aa), and ORF3 encodes a small protein of only 113-114 aa that is required for virion egress [53]. Four genotypes (1-4) of HEV have been identified in mammalian species [33]. Genotype 1 and 2 HEVs are restricted to humans and are often associated with large outbreaks and epidemics in developing countries. Genotype 3 and 4 HEVs are believed to undergo zoonotic transmission, with a reservoir in pigs and, possibly, a range of other mammals, and are responsible for sporadic cases of hepatitis E in both developing and industrialized countries [4, 22, 32, 34, 51, 54]. Genotype 3 is distributed worldwide except for Africa, while genotype 4 is distributed mainly in Asian countries including China, India, Indonesia, Japan, and Vietnam [33].

The uniqueness of HEV is that, among the five known hepatitis viruses (A to E), HEV is the only one with animal reservoirs. Swine HEV was first isolated from pigs and genetically characterized in 1997 [21], and then an avian HEV was identified and characterized from chickens with hepatitis-splenomegaly syndrome in 2001 [9]. In addition, HEV strains have recently been genetically identified in wild boars, deer, mongooses, rabbits, and rats [13, 28, 40, 42, 55], which have significantly broadened the host range and genomic diversity of HEV.

Regarding HEV from wild boars, HEV RNA and antibodies have been detected in several European countries, Australia, and Japan, with a seroprevalence rate of 9-43% and an HEV RNA detection rate of 2-25% [2, 5, 14, 18, 23, 30, 37, 40]. Transmission of HEV from wild boars to humans has been reported, particularly in Japan [15, 20, 39, 50]. However, a nationwide survey of HEV infection among wild boars in Japan has not been conducted thus far, and it remains unknown how frequently HEV infection is occurring among wild boars throughout Japan. Therefore, in an attempt to investigate the prevalence of HEV infection among wild boars in Japan stratified by year of capture and geographic region, antibodies to HEV (anti-HEV) and HEV RNA were assayed in serum samples and/or liver specimens obtained from 578 wild boars.

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## Materials and methods

### Serum and liver samples from wild boars

Paired serum and liver specimens, serum only, or liver tissues only were obtained from a total of 578 wild boars (*Sus scrofa leucomystax*) that had been captured in 18 prefectures (located from north to south in Japan): Ibaraki (91 boars), Tochigi (23), Saitama (13), Kanagawa (9), Toyama (17), Ishikawa (7), Fukui (25), Yamanashi (8), Nagano (48), Gifu (100), Shizuoka (8), Shiga (6), Kyoto (4), Nara (8), Wakayama (2), Tottori (19), Okayama (65), and Yamaguchi (19) on mainland Honshu, the prefectures of Kagawa (39) and Tokushima (26) on Shikoku Island, and the prefectures of Saga (2), Nagasaki (11), Kumamoto (6), Oita (14), and Miyazaki (8) on Kyushu Island (Supplementary Figure 1) between January 2003 and March 2010. A total of 507 serum samples and 552 liver tissues including 481 paired serum and liver specimens were available from the 578 boars: the 578 boars in the present study included the 128 boars reported in our previous studies [30, 40].

## ELISA for detecting anti-HEV antibodies

To detect anti-HEV IgG or IgA in serum samples from wild boars, an enzyme-linked immunosorbent assay (ELISA) was performed using purified recombinant ORF2 protein from the HE-J1 strain (genotype 4) that had been expressed in silkworm pupae [25] as described previously [46]. Briefly, ELISA microplates (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated with the recombinant ORF2 protein (250 ng/well). The samples were added to each well of the microplates at a dilution of 1:100 in 10 mM Tris-buffered saline containing 40% Block Ace (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), 0.18% Tween 20, and a mock protein [optical density (OD) at 280 nm = 0.1] that had been obtained from the pupae of silkworms infected with nonrecombinant baculovirus. As enzyme-labeled antibodies, the peroxidase-conjugated rabbit IgG fraction to swine IgG (whole molecule) (MP Biomedicals, LLC., Solon, OH) was used for the boar anti-HEV IgG assay, and peroxidase-conjugated goat IgG against porcine IgA (Serotec Ltd., Oxford, UK) was used for the swine anti-HEV IgA assay. The OD of each sample was read at 450 nm. To determine the cutoff values in the anti-HEV IgG and IgA assays, 118 control swine serum samples that were exclusively negative for HEV RNA [45] were used as a panel. The OD values of 0.274 (mean + 6 standard deviations) and 0.303 (mean + 6 standard deviations) were used as the cutoff values for the swine anti-HEV IgG and IgA assays, respectively [46]. Test samples with OD values equal to or greater than the respective cutoff values were considered to be positive for anti-HEV IgG or IgA. The specificity of the anti-HEV assays was verified by absorption with the same recombinant ORF2 protein that was used as the antigen probe. Briefly, if the OD value of the tested sample was less than 30% of the original value after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV IgG or IgA.

## Detection of HEV RNA

Reverse transcription (RT)-polymerase chain reaction (PCR) was performed for detection of HEV RNA. Total RNA was extracted from 100 μl of each serum sample using TRIzol LS Reagent (Invitrogen, Tokyo, Japan), or 50 mg of each liver specimen using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The extracted RNA was reverse-transcribed with SuperScript II (Invitrogen), and subsequent nested PCR (ORF2-457 PCR) was performed with primers derived from the areas of the ORF2 region that are well conserved across all four genotypes, using a method described previously [25]. The size of the amplification product of the first-round PCR was 506 bp, and that of the amplification product of the second-round PCR was 457 bp. To confirm the presence of HEV RNA, another nested RT-PCR (ORF1-459 PCR) with primers targeting the 5'UTR and 5'-terminus of ORF1 gene [25], capable of amplifying all four known genotypes of HEV strains reported thus far, was carried out. The size of the amplification product of the first-round PCR was 567 bp, and that of the amplification product of the second-round PCR was 459 bp. The specificity of the two RT-PCR assays was verified by sequence analysis as described below. The sensitivity of the RT-PCR assay was assessed as described previously [25, 45].

## Quantitation of HEV RNA

HEV RNA was quantitated by real-time detection RT-PCR according to a previously described method [47] with slight modifications. In brief, total RNA extracted from 100 μl of the diluted serum sample or 50 mg of liver tissue was subjected to real-time RT-PCR using a QuantiTect Probe RT-PCR Kit (QIAGEN, Tokyo, Japan), with the sense primer HE311 (5'-GGT GGT TTC TGG GGT GAC-3'), antisense primer HE312 (5'-AGG GGT TGG TTG GAT GAA-3'), and a probe (HE313-P) consisting of an

**Table 1** Prevalence of anti-HEV IgG and HEV RNA in wild boars in Japan, stratified by year of capture

| Year | No. of boars tested | Serum | | | Liver | | No. of HEV RNA-positive boars |
|---|---|---|---|---|---|---|---|
| | | No. | Anti-HEV IgG-positive | HEV RNA-positive | No. | HEV RNA-positive | |
| 2003 | 20 | 20 | 1 (5.0%) | 1 (5.0%) | 13 | 1 (7.7%) | 1 (5.0%) |
| 2004 | 52 | 46 | 3 (6.5%) | 1 (2.2%) | 38 | 1 (2.6%) | 1 (1.9%) |
| 2005 | 84 | 46 | 5 (10.9%) | 0 | 82 | 4 (4.9%) | 4 (4.8%) |
| 2006 | 117 | 97 | 5 (5.2%) | 3 (3.1%) | 117 | 6 (5.1%) | 6 (5.1%) |
| 2007 | 92 | 90 | 5 (5.6%) | 1 (1.1%) | 92 | 2 (2.2%) | 2 (2.2%) |
| 2008 | 67 | 66 | 4 (6.1%) | 2 (3.0%) | 67 | 2 (3.0%) | 2 (3.0%) |
| 2009 | 112 | 109 | 11 (10.1%) | 2 (1.8%) | 109 | 2 (1.8%) | 2 (1.8%) |
| 2010 | 34 | 33 | 7 (21.2%) | 1 (3.0%) | 34 | 1 (2.9%) | 1 (2.9%) |
| Total | 578 | 507 | 41 (8.1%) | 11 (2.2%) | 552 | 19 (3.4%) | 19 (3.3%) |

oligonucleotide with a 5'-reporter dye (FAM) and a 3'-quencher dye (TAMRA) (5'-FAM-TGA TTC TCA GCC CTT CGC-TAMRA-3') on a LightCycler apparatus (Roche Diagnostics K.K., Tokyo, Japan). The thermal cycler conditions were 50°C for 20 min, 95°C for 15 min, and 45 cycles of 95°C for 1 s and 60°C for 60 s. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate, and the mean value was adopted.

Amplification of the full-length HEV genome

Total RNA was extracted from 900 μl each of two serum samples obtained from a viremic wild boar and a patient with sporadic hepatitis E and subjected to cDNA synthesis followed by nested PCR of nine overlapping regions including the extreme 5'- and 3'-terminal regions: the amplified regions excluding the primer sequences were nucleotides (nt) 1-60 (60 nt), nt 37-1199 (1,163 nt), nt 1069-3127 (2,059 nt), nt 3014-4588 (1,575 nt), nt 4384-5310 (927 nt), nt 5236-5983 (748 nt), nt 5699-6381 (683 nt), nt 6343-7107 (765 nt) and nt 6824-7225 (402 nt) for the two isolates obtained. The extreme 5'-end sequence (nt 1-60) was determined by a modified rapid amplification of cDNA ends (RACE) technique called RNA ligase-mediated RACE (RLM-RACE) with the First Choice RLM-RACE kit (Ambion, Austin, TX), as described previously [31]. Amplification of the 3'-end sequence (nt 6824-7225; 402 nt excluding the poly (A) tail) was performed by the RACE method as described previously [31].

Sequence analysis of PCR products

The amplification product was sequenced directly on both strands or after cloning into a plasmid vector, using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using the Genetyx ver.10 software program (Genetyx Corp., Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ: National Institute of Genetics,

**Table 2** Prevalence of anti-HEV IgG and HEV RNA among wild boars in Japan, stratified by geographic region

| Region | No. of boars tested | Serum | | | Liver | | No. of HEV RNA-positive boars (%) |
|---|---|---|---|---|---|---|---|
| | | No. | Anti-HEV IgG-positive (%) | HEV RNA-positive (%) | No. | HEV RNA-Positive (%) | |
| Ibaraki | 91 | 89 | 2 (2.2) | 0 | 91 | 0 | 0 |
| Tochigi | 23 | 20 | 0 | 0 | 23 | 0 | 0 |
| Saitama | 13 | 12 | 0 | 0 | 13 | 0 | 0 |
| Kanagawa | 9 | 9 | 0 | 0 | 9 | 0 | 0 |
| Toyama | 17 | 15 | 0 | 0 | 17 | 0 | 0 |
| Ishikawa | 7 | 7 | 0 | 0 | 7 | 0 | 0 |
| Fukui | 25 | 24 | 3 (12.5) | 2 (8.3) | 24 | 2 (8.3) | 2 (8.0) |
| Yamanashi | 8 | 0 | NA | NA | 8 | 2 (25.0) | 2 (25.0) |
| Nagano | 48 | 33 | 2 (6.1) | 1 (3.0) | 48 | 1 (2.1) | 1 (2.1) |
| Gifu | 100 | 99 | 8 (8.1) | 4 (4.0) | 98 | 4 (4.1) | 4 (4.0) |
| Shizuoka | 8 | 2 | 2 (100) | 0 | 8 | 2 (25.0) | 2 (25.0) |
| Shiga | 6 | 4 | 3 (75.0) | 0 | 5 | 1 (20.0) | 1 (16.7) |
| Kyoto | 4 | 2 | 0 | 0 | 4 | 0 | 0 |
| Nara | 8 | 8 | 4 (50.0) | 1 (12.5) | 8 | 1 (12.5) | 1 (12.5) |
| Wakayama | 2 | 2 | 0 | 0 | 2 | 0 | 0 |
| Tottori | 19 | 16 | 0 | 0 | 6 | 0 | 0 |
| Okayama | 65 | 57 | 7 (12.3) | 1 (1.8) | 65 | 3 (4.6) | 3 (4.6) |
| Yamaguchi | 19 | 2 | 0 | 0 | 19 | 1 (5.3) | 1 (5.3) |
| Kagawa | 39 | 39 | 5 (12.8) | 0 | 39 | 0 | 0 |
| Tokushima | 26 | 26 | 1 (3.8) | 1 (3.8) | 19 | 1 (5.3) | 1 (3.8) |
| Saga | 2 | 2 | 0 | 1 (50.0) | 2 | 1 (50.0) | 1 (50.0) |
| Nagasaki | 11 | 11 | 3 (27.3) | 0 | 11 | 0 | 0 |
| Kumamoto | 6 | 6 | 0 | 0 | 4 | 0 | 0 |
| Oita | 14 | 14 | 0 | 0 | 14 | 0 | 0 |
| Miyazaki | 8 | 8 | 1 (12.5) | 0 | 8 | 0 | 0 |
| Total | 578 | 507 | 41 (8.1) | 11 (2.2) | 552 | 19 (3.4) | 19 (3.3) |

*NA* not applicable

Mishima, Japan) [11]. Phylogenetic trees were constructed by the PHYML method version 3.0 [8] implemented via the web server PALM (http://palm.iis.sinica.edu.tw) [3], based on the 412-nt ORF1 or ORF2 sequence, or the full-length sequence. Maximum-likelihood phylogenetic trees were constructed using the best model selected by MODELTEST version 3.7 [35] under Akaike information criterion (AIC). The final trees were visualized using the FigTree program version 1.2.3 (http://tree.bio.ed.ac.uk/software/figtree/).

## Results

### The prevalence of anti-HEV antibodies and HEV RNA in wild boars in Japan

Serum samples obtained from 507 wild boars and liver tissues from 552 boars were tested for the presence of anti-HEV antibodies and HEV RNA. Overall, 41 boars (8.1%) were positive for anti-HEV IgG, with the prevalence differing by year of capture, ranging from 5.0 % in 2003 to 21.2% in 2010, and 19 boars (3.3%) had detectable HEV RNA in serum and/or liver specimens, with the prevalence differing by year of capture, ranging from 1.8% in 2009 to 5.1% in 2006 (Table 1). The anti-HEV IgG was detectable in wild boars captured in 12 (48.0%) of the 25 prefectures studied (Table 2). Among the 12 prefectures with anti-HEV IgG-positive boars, the prevalence of boar anti-HEV IgG differed markedly by geographic region (prefecture), from 2.2% to 100% (median, 12.5%) (2.2-27.3%, when restricted to prefectures from which 11 or more serum samples were tested). Boars with ongoing HEV infection were found in 11 prefectures (44.0%), with the prevalence ranging from 2.1% to 50% (median, 8.0%) (2.1-8.0% when restricted to prefectures in which 11 or more boars were tested).

### Characteristics of the 19 wild boars that were positive for HEV RNA in serum and/or liver specimens

Of the 12 boars whose serum samples were available, eight tested positive for anti-HEV IgG, with an OD value of 0.567-2.771, and five boars had anti-HEV IgA in their serum, with an OD value of 0.436-1.486 (Table 3), despite the fact that only two of the 495 boars testing negative for HEV RNA in their serum had detectable anti-HEV IgA (41.7% vs. 0.4%, $P < 0.0001$ [$\chi^2$-test]). HEV RNA was detectable in the serum samples of 11 of the 12 boars tested, although the titer was low ($\leq 7.9 \times 10^4$ copies/ml).

**Table 3** Characteristics of the 19 wild boars with ongoing HEV infection

| ID. no. | Date of sampling | Body weight (kg) | Region | Anti-HEV ($OD_{450}$) in serum[a] | | HEV RNA titer | | HEV genotype | HEV isolate name |
|---|---|---|---|---|---|---|---|---|---|
| | | | | IgG-class | IgA-class | copies/ml (serum) | copies/mg (liver) | | |
| WB008 | '03.12.19 | 60 | Saga | 0.272 (−) | 0.045 (−) | <122 (+) | <400 (+) | 3 | wbJSG_03 |
| WB047 | '04.12.09 | 50 | Tokushima | 0.645 (+) | 0.032 (−) | <122 (+) | <400 (+) | 3 | wbJTS_04 |
| WB092 | '05.02.06 | 50 | Yamaguchi | NA | NA | NA | $2.8 \times 10^6$ | 3 | wbJYG_05 |
| WB137 | '06.01.12 | 20 | Okayama | NA | NA | NA | <400 (+) | Unclassified | wbJOY_06 |
| WB169 | '05.11.19 | 150 | Shizuoka | NA | NA | NA | <400 (+) | 3 | wbJSO_05-1 |
| WB170 | '05.11.19 | 10 | Shizuoka | NA | NA | NA | $6.0 \times 10^5$ | 3 | wbJSO_05-2 |
| WB177 | '05.12.31 | 30 | Shiga | NA | NA | NA | $1.0 \times 10^4$ | 3 | wbJSH_05 |
| WB185 | '06.02.12 | 110 | Fukui | 0.056 (−) | 0.018 (−) | $2.0 \times 10^2$ | <400 (+) | 3 | wbJFI_06 |
| WB190 | '06.01.22 | Unknown | Yamanashi | NA | NA | NA | $4.8 \times 10^5$ | 3 | wbJYN_06-1 |
| WB191 | '06.01.23 | 240 | Yamanashi | NA | NA | NA | $2.3 \times 10^5$ | 3 | wbJYN_06-2 |
| WB247 | '07.01.09 | 15 | Okayama | 0.986 (+) | 0.094 (−) | <122 (+) | $1.3 \times 10^4$ | 3 | wbJOY_07-1 |
| WB249 | '07.01.09 | 15 | Okayama | 0.567 (+) | 0.030 (−) | (−) | $4.4 \times 10^5$ | 3 | wbJOY_07-2 |
| WB254 | '06.12.23 | 15 | Gifu | 1.956 (+) | 1.486 (+) | $1.3 \times 10^3$ | $7.5 \times 10^4$ | 4 | wbJGF_06-1 |
| WB255 | '06.12.23 | 31 | Gifu | 1.553 (+) | 0.519 (+) | <122 (+) | <400 (+) | 4 | wbJGF_06-2 |
| WB401 | '08.09.14 | 27 | Gifu | 2.771 (+) | 1.396 (+) | $3.8 \times 10^4$ | $4.8 \times 10^6$ | 4 | wbJGF_08-1 |
| WB405 | '08.09.16 | 25 | Gifu | 0.187 (−) | 0.042 (−) | <122 (+) | <400 (+) | 4 | wbJGF_08-2 |
| WB518 | '10.01.22 | 20 | Nara | 2.245 (+) | 0.436 (+) | $8.7 \times 10^3$ | $1.6 \times 10^6$ | 3 | wbJNR_10 |
| WB533 | '09.12.19 | 80 | Nagano | 0.061 (−) | 0.040 (−) | <122 (+) | <400 (+) | 3 | wbJNN_09 |
| WB557 | '09.11.19 | Unknown | Fukui | 1.629 (+) | 0.487 (+) | $7.9 \times 10^4$ | <400 (+) | 3 | wbJFI_09 |

*NA* not available

[a] The cutoff values for the anti-HEV IgG and anti-HEV IgA are 0.274 and 0.303, respectively

**Table 4** Comparison of the identity (%) within the 412-nt ORF1 or ORF2 sequence of 19 boar HEV isolates obtained in the present study with reported HEV isolates whose complete or nearly complete sequence is known

| HEV isolate | No. of isolates compared | Identity (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Genotype 3 (n = 14)[a] | | Genotype 4 (n = 4)[a] | | Unclassified (n = 1)[a] | |
| | | ORF1 (412 nt) | ORF2 (412 nt) | ORF1 (412 nt) | ORF2 (412 nt) | ORF1 (412 nt) | ORF2 (412 nt) |
| Mammalian | 137 | 75.0–95.9 (83.2 ± 5.1) | 73.8–95.1 (82.5 ± 4.9) | 75.7–99.0 (82.2 ± 4.6) | 76.0–99.0 (83.1 ± 4.6) | 75.0–81.6 (79.1 ± 1.3) | 75.7–82.0 (79.4 ± 1.4) |
| Genotype 1 | 19 | 75.0–79.4 (76.9 ± 0.9) | 75.5–81.6 (78.7 ± 1.1) | 76.5–79.9 (78.4 ± 0.7) | 79.1–82.8 (81.0 ± 0.9) | 76.7–79.6 (78.3 ± 0.9) | 75.7–79.1 (77.2 ± 0.9) |
| Genotype 2 | 1 | 76.2–79.1 (77.5 ± 1.0) | 73.8–78.2 (76.0 ± 1.6) | 80.3–81.1 (80.5 ± 0.4) | 76.0–77.2 (76.8 ± 0.5) | 79.1 | 76.0 |
| Genotype 3 | 63 | 77.9–95.9 (87.9 ± 3.7) | 77.4–95.1 (87.0 ± 3.6) | 75.7–81.8 (79.0 ± 1.2) | 76.0–82.3 (79.5 ± 1.3) | 75.0–80.3 (78.4 ± 1.0) | 76.7–82.0 (79.6 ± 1.1) |
| Human | 32 | 81.1–95.9 (89.3 ± 3.2) | 81.6–95.1 (88.3 ± 3.1) | 77.7–81.8 (79.0 ± 1.1) | 77.4–82.3 (80.1 ± 1.1) | 76.2–79.9 (78.3 ± 0.8) | 76.9–82.0 (79.7 ± 1.1) |
| Swine | 22 | 81.3–94.2 (86.5 ± 2.9) | 80.3–93.4 (85.8 ± 2.9) | 76.5–81.6 (79.4 ± 1.1) | 76.2–80.6 (79.0 ± 0.8) | 76.7–80.3 (78.7 ± 0.9) | 77.4–81.1 (79.6 ± 1.1) |
| Wild boar | 4 | 82.6–94.4 (87.3 ± 3.2) | 82.3–93.2 (86.4 ± 3.0) | 76.7–79.1 (78.0 ± 0.8) | 77.2–81.1 (78.3 ± 1.5) | 78.5–80.3 (79.4 ± 0.8) | 79.4–79.9 (79.5 ± 0.2) |
| Deer | 1 | 85.7–94.7 (92.1 ± 2.6) | 86.7–93.2 (90.8 ± 2.2) | 77.9–78.2 (78.0 ± 0.1) | 80.6–81.1 (80.7 ± 0.2) | 79.4 | 79.4 |
| Mongoose | 1 | 87.6–95.4 (92.5 ± 1.8) | 86.9–94.9 (90.5 ± 1.9) | 78.2–78.4 (78.3 ± 0.1) | 79.9–80.3 (80.0 ± 0.2) | 77.9 | 77.9 |
| Rabbit | 3 | 77.9–84.0 (80.9 ± 1.9) | 77.4–83.5 (80.5 ± 1.4) | 75.7–78.9 (77.4 ± 1.3) | 76.0–78.6 (77.7 ± 1.0) | 75.0–77.7 (76.7 ± 1.5) | 76.7–79.1 (77.8 ± 1.2) |
| Genotype 4 | 54 | 76.9–82.0 (80.1 ± 1.0) | 76.0–82.3 (78.8 ± 1.0) | 84.0–99.0 (87.4 ± 2.9) | 83.3–99.0 (88.3 ± 2.6) | 77.7–81.6 (80.2 ± 1.0) | 77.7–82.0 (80.1 ± 0.9) |
| Human | 39 | 76.9–82.0 (80.3 ± 1.0) | 76.7–82.3 (78.9 ± 0.9) | 84.0–99.0 (87.6 ± 3.2) | 85.4–99.0 (88.6 ± 2.6) | 78.4–81.6 (80.4 ± 0.9) | 78.4–82.0 (80.3 ± 0.8) |
| Swine | 15 | 77.7–81.8 (79.7 ± 1.0) | 76.0–81.3 (78.6 ± 1.2) | 84.2–91.3 (86.9 ± 1.8) | 83.3–92.5 (87.5 ± 2.4) | 77.7–81.6 (79.6 ± 1.1) | 77.7–80.8 (79.4 ± 0.9) |
| Avian | 4 | 53.4–57.0 (55.4 ± 0.9) | 54.9–58.7 (56.8 ± 1.0) | 57.3–59.2 (57.9 ± 0.6) | 55.8–61.2 (58.6 ± 1.9) | 57.3–60.0 (58.3 ± 1.2) | 55.6–57.5 (57.0 ± 1.0) |

[a] See Table 3

Despite this low HEV RNA titer in the liver specimens (<400 copies/mg) in nine boars, it was high ($1.0 \times 10^4$ to $4.8 \times 10^6$ copies/mg) in the remaining 10 boars.

Genetic heterogeneity of boar HEV isolates recovered from wild boars in Japan

The amplification products of ORF1 (412 nt; primer sequences at both ends excluded) and ORF2 (412 nt) from 19 HEV-infected boars were sequenced and compared (Table 4). Among the 19 boar HEV isolates obtained from the infected boars, 14 isolates (74%) were closely related to genotype 3 HEV isolates (n = 63), with nucleotide sequence identities of 77.7-95.9 (87.9 ± 3.7)% within ORF1 and 77.4-95.1 (87.0 ± 3.6)% within ORF2, but were only 75.0-82.0 (79.2 ± 1.7)% (ORF1) and 73.8-82.3 (78.7 ± 1.1)% (ORF2) similar to known HEV isolates of other genotypes (1, 2, and 4) (n = 74). These 14 isolates shared identities of 85.0-100 (91.2 ± 3.1)% within the 412-nt ORF1 sequence and 85.9-100 (90.9 ± 2.9)% within the 412-nt ORF2 sequence with each other. Four isolates were 84.0-99.0 (87.4 ± 2.9) and 83.3-99.0 (88.3 ± 2.6)% similar to genotype 4 HEV isolates (n = 54) within ORF1 and ORF2, respectively, but were only 75.7-81.8 (78.9 ± 1.1)% (ORF1) and 76.0-82.8 (79.8 ± 1.4)% (ORF2) similar to known HEV isolates of the other three genotypes (n = 83). These four isolates shared identities of 98.3-100 (98.9 ± 0.6)% within the 412-nt ORF1 sequence and 98.1-100 (98.5 ± 0.8)% within the 412-nt ORF2 sequence with each other. These results indicate that 14 and 4 Japanese boar HEV isolates obtained in the present study were classifiable as genotype 3 and genotype 4, respectively. Of interest, however, the remaining isolate (wbJOY_06) was only 75.0-81.6 (79.1 ± 1.3)% and 75.7-82.0 (79.4 ± 1.4)% identical to all known HEV isolates of genotypes 1-4 (n = 137) within ORF1 and ORF2, respectively, suggesting that the wbJOY_06 isolate belongs to a previously unidentified genotype.

The phylogenetic trees constructed by the PHYML method using the GTR + R substitution model, based on the 412-nt ORF1 sequence (Fig. 1A) and 412-nt ORF2 sequence (Fig. 1B), confirmed that 18 of the 19 boar HEV isolates obtained in the present study belonged to genotype 3 or 4 and that the remaining isolate (wbJOY_06) was markedly different from all known HEV isolates of the four genotypes 1-4 (see Supplementary Table 1).

Comparison of HEV isolates obtained from wild boars in the present study with those of human origin

The 14 genotype 3 HEV isolates obtained in the present study shared the highest identity of 92.0-95.9% within the 412-nt ORF1 sequence and 91.5-99.0% within the 412-nt ORF2 sequence with HEV isolates recovered from Japanese patients who contracted domestic HEV infection. On the other hand, the four genotype 4 HEV isolates obtained in Gifu Prefecture in the present study shared the highest identity of 99.0-99.8% within the 412-nt ORF1 sequence and 98.8-99.8% within the 412-nt ORF2 sequence, with HEV isolates recovered from hepatitis patients living in Aichi Prefecture who contracted the HEV infection after consuming undercooked meat and/or the liver of wild boars that had been caught in Aichi Prefecture [39], which borders Gifu Prefecture in the north (Supplementary Table 2).

To further characterize HEV isolates that might be implicated in zoonotic food-borne transmission, the complete genomic sequences were determined for wbJGF_08-1, which had the highest load of HEV in serum among those from the four boars captured in Gifu (Table 3), and HE-Aichi-C1, which shared the highest identity of 99.8% with wbJGF_08-1 in the 412-nt ORF1 sequence. The wbJGF_08-1 and HE-Aichi-C1 isolates each had a genomic length of 7,225 nt, excluding the poly(A) tract at the 3' terminus, and possessed three major ORFs similar to other reported human and animal HEV isolates [21, 33, 49]. Based on the evidence that ORF2 and ORF3 proteins are translated from a single bicistronic subgenomic RNA [7], each isolate was presumed to encode an ORF1 protein of 1,702 aa (nt 26-5131), an ORF2 protein of 660 aa (nt 5173-7152), and an ORF3 protein of 114 aa (nt 5159-5500). The 5'UTR of the wbJGF_08-1 and HE-Aichi-C1 genomes were each comprised of 25 nt. The 3'UTR sequence of wbJGF_08-1 and HE-Aichi-C1 each consisted of 73 nt (nt 7153-7225), excluding the poly(A) tail. The wbJGF_08-1 and HE-Aichi-C1 isolates showed an overall nucleotide identity of 98.6% (7,122/7,225). The nucleotide sequences of the 5'UTRs of the two isolates were 100% similar to each other and to the reported genotype 4 isolates [44, 52]. The nucleotide sequence of the 3'UTR of the two isolates differed by 2 nt within the 73-nt sequence, although this region showed appreciable diversity among the current two isolates and the 49 HEV isolates of genotype 4 whose extreme 3' end sequence is known, differing by up to 37.3%. The wbJGF_08-1 and HE-Aichi-C1 isolates were 98.5 (5,030/5,106)%, 98.7 (1,955/1,980)%, and 98.8 (338/342)% similar to each other in their nucleotide sequence of ORF1, ORF2, and ORF3, respectively. At the amino acid sequence level, the two isolates were 99.4 (1,692/1,702)%, 99.5 (657/660)%, and 99.1 (113/114)% identical to each other in ORF1, ORF2, and ORF3, respectively.

Comparison of the wbJGF_08-1 and HE-Aichi-C1 genomes against 137 reported HEV genomes of genotypes 1-4 whose entire or nearly entire nucleotide sequence is known revealed that they were most closely related to the JKS-Shiz07L and JYN-Shiz08L isolates (AB521805-

**Fig. 1** Maximum-likelihood phylogenetic trees of the 412-nt sequences within ORF1 (**A**) and ORF2 (**B**) of 137 reported HEV isolates of genotypes 1-4 obtained from humans, domestic swine, wild boars, wild deer, mongooses, and rabbits for which the complete or nearly complete sequence is known (001-137: see Supplementary Table 1) and 19 boar isolates obtained in the present study, which are indicated in boldface type for clarity. The trees were constructed using PHYML (model GTR + G), with optimized tree topology and branch lengths, and the numbers associated with tree branches are indicative of the percentage of 100 full maximum-likelihood bootstrap replicates that support the existence of the branches

**Fig. 1** continued

AB521806), genotype 4 human HEV isolates recovered from hepatitis patients who lived in Shizuoka Prefecture bordering Aichi Prefecture (Supplementary Figure 1), with identities of 98.5-99.2%. However, they were only 83.4-83.5% similar to the prototype genotype 4 HEV isolate (T1) of Chinese origin [52] in the nucleotide sequence of

the full genome. In contrast, the wbJGF_08-1 and HE-Aichi-C1 isolates were only 73.1-76.2% similar to the other human and animal HEV isolates of genotypes 1-3. A phylogenetic tree constructed by the PHYML method (GTR + I + G substitution model) based on the full genomic sequences confirmed that wbJGF_08-1 and HE-Aichi-C1 belong to genotype 4 and are most closely related to JKS-Shiz07L and JYN-Shiz08L (Fig. 2).

## Discussion

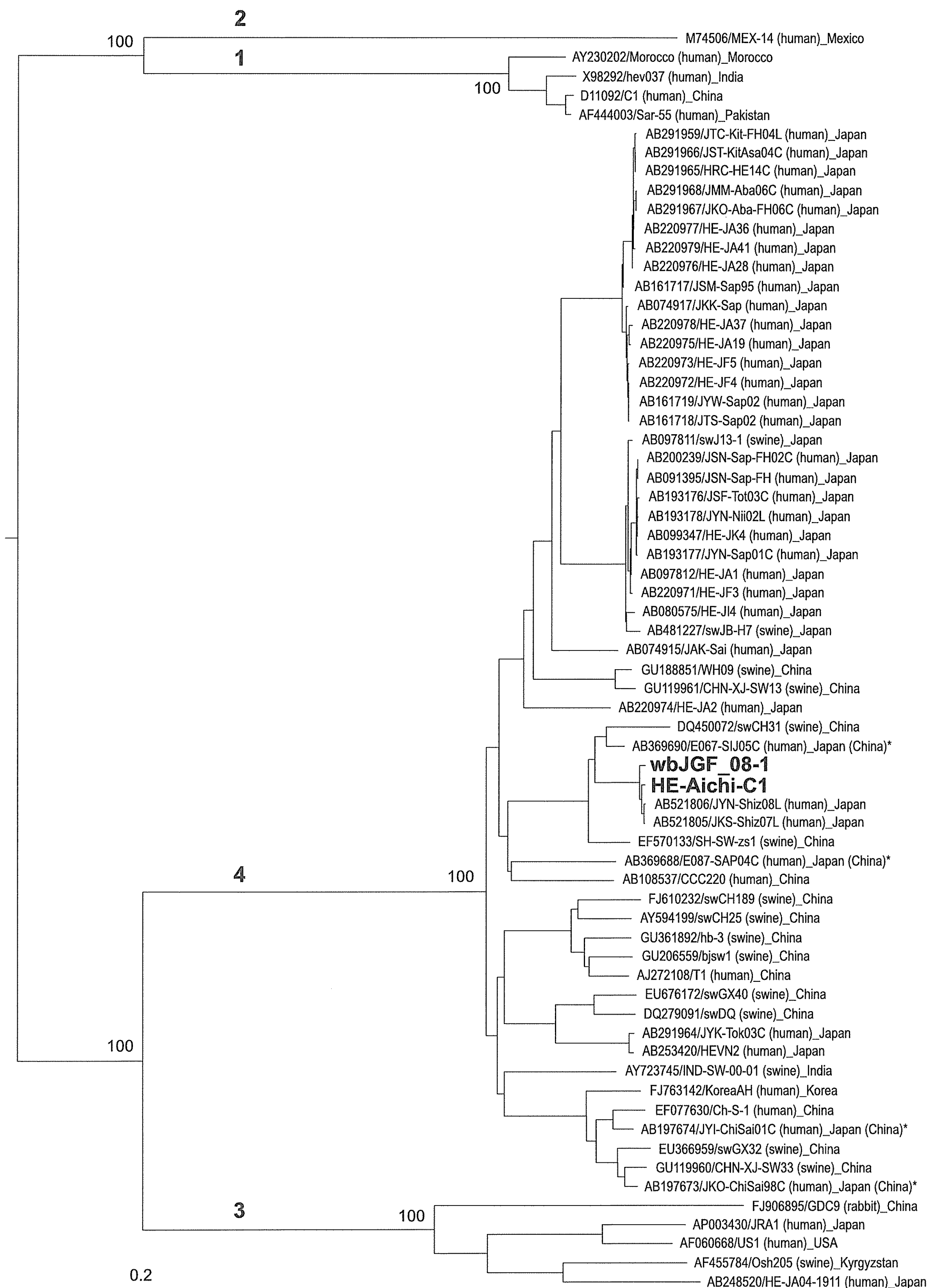
Wild boars live mainly in the southern part of Japan, including 42 of the 47 prefectures located in the central and southern parts of Honshu Island, Shikoku and Kyushu Islands, and Okinawa. So far, the prevalence of HEV infection in wild boars has been reported from only limited areas, including four prefectures (Gunma, Aichi, Ehime, and Okinawa) [12, 23, 27, 38]. In this study, we assessed the nationwide prevalence of HEV in wild boars captured in 25 (60%) of the 42 prefectures with boar habitats from 2003-2010. The results showed that the overall prevalence of anti-HEV IgG and HEV RNA was 8.1% and 3.3%, respectively, in the boars studied in Japan and was within the range of the previously reported prevalence of anti-HEV IgG (4.5-25.5%) and HEV RNA (1.1-13.3%), respectively [12, 23, 27, 38]. Although it was evident that the prevalence of anti-HEV IgG and HEV RNA differed by geographic region (prefecture) in the present study (Table 2) and the prevalence of anti-HEV IgG and HEV RNA among wild boars also differed by year of capture (Table 1), taken together with the previously reported results, the prevalence of anti-HEV IgG and HEV in wild boars throughout Japan is presumed to be 14.7% (145/988) and 3.9% (45/1,165), respectively. Of note, in 10 (40%) of the 25 prefectures studied, none of the boars tested had anti-HEV IgG or HEV RNA. However, we cannot rule out the possibility that HEV does circulate among wild boars even in these 10 prefectures, because the number of boars captured in each prefecture was only 2-23 (median, 11). To draw a plausible conclusion about the presence or absence of prefectures where wild boars are free of HEV infection in Japan, a larger number of boars in each prefecture must be examined in future studies.

In Japan, HEV has been recognized to be an important causative agent of sporadic acute hepatitis of non-A, non-B, and non-C etiology [25, 32]. For patients who are infected with HEV domestically in this country, transmission via blood transfusion undoubtedly occurs [19, 24]. However, a significantly more important transmission route is zoonotic, from swine, wild boars and wild deer [16, 51, 54]. Polyphyletic strains of HEV have been recovered from patients with autochthonous hepatitis E and domestic pigs, and these segregate into genotypes 3 and 4 [25, 41]. The present study revealed that HEV strains circulating in wild boars in Japan belong to at least two genotypes (genotypes 3 and 4) and are also highly divergent, even within the same genotype, similar to what is observed in humans and domestic pigs in Japan, but clearly different from what is seen in European countries, where only HEV genotype 3 prevails in wild boars [5, 14, 18, 34, 37],

Although only genotype 3 HEV strains have thus far been isolated from wild deer and a mongoose, genotype 3 and 4 HEV strains have been recovered from domestic pigs and wild boars in Japan. Swine HEV strains of genotype 4 have been isolated in China, India, and Japan, and their entire or nearly entire genomic sequences have been determined for 16 strains (13 from China, 2 from Japan, and 1 from India) and deposited in the DNA databases as of December 2010. However, genotype 4 boar HEV strains have been isolated only in Japan, restricted to Aichi [12], Okinawa [27], and Gifu (this study) Prefectures. Since no boar HEV strains of genotype 4 whose entire genomic sequence has been determined were available, we determined the full-length genomic sequence of one (wbJGF_08-1) of the four genotype 4 boar isolates identified in the present study in Gifu Prefecture, which had the highest HEV load in serum, and a human HEV isolate of the same genotype (HE-Aichi-C1) that was 99.8% identical to wbJGF_08-1 within the 412-nt ORF1 sequence. The HE-Aichi-C1 was recovered from a 48-year-old male patient living in Aichi Prefecture, adjacent to Gifu Prefecture in the north, who had consumed undercooked wild boar meat approximately 30 days before the onset of acute hepatitis E [39]. Of note, wbJGF_08-1 and HE-Aichi-C1 shared 98.6% identity over the entire genome, similar to the finding that a pair of swine and human HEV strains of genotype 4 with 99.0% identity over the entire genome was present in a restricted area in Hokkaido [29], supporting the notion that the wild boar is an important reservoir of genotype 3 HEV [15, 20, 50] and thus suggesting that wild boar is also a reservoir of genotype 4 HEV.

The wild boars with HEV RNA in serum and/or liver specimens, representing ongoing HEV infection, had a body weight of 10-240 kg, thus indicating that not only immature boars, but also adult boars, can acquire HEV infection. Although it is known that 2- to 4-month-old domestic pigs are highly viremic for HEV and those aged ≥6 months rarely have ongoing HEV infection [45], it seems likely that even domestic pigs aged ≥6 months can contract an HEV infection if they are exposed to HEV at older ages. In support of this conjecture, it has been reported that a small fraction of pigs (7% or 3/43) at the finishing stage (5-6 months of age) still shed HEV [26].

It was interesting to note that, in the present study, a boar HEV isolate (wbJOY_06) that is remotely related to




all known HEV isolates of genotypes 1-4 and may be classifiable into a novel genotype was identified. The wbJOY_06 strain was recovered from the liver tissues of a 20-kg wild boar that had been captured in Okayama Prefecture, located in the western part of Honshu Island (Supplementary Figure 1), in January 2006 as one of 10 boars caught during the same period. The remaining nine boars were negative for HEV RNA, but one boar had

◀ **Fig. 2** Maximum-likelihood phylogenetic tree of the entire genomic sequence of 64 reported HEV isolates, including 10 representative HEV isolates of genotypes 1-3 and all 54 genotype 4 HEV isolates available from DNA databases as of December 2010, and two genotype 4 isolates (wbJGF_08-1 and HE-Aichi-C1) obtained in the present study, which are indicated in boldface type for clarity. The tree was constructed by the PHYML method using the GTR + I + G substitution model, with optimized tree topology and branch lengths, and the numbers associated with tree branches are indicative of the percentage of 10 full maximum-likelihood bootstrap replicates that support the existence of the branches. Each reported isolate is indicated by the accession number followed by isolate name after the slash, human or other host animal in parentheses, and the name of the country where the HEV strain was isolated. *Country names in parentheses denote the country where the patient was presumed to have contracted HEV infection while traveling

detectable anti-HEV IgG, suggesting the circulation of a wbJOY_06-like virus in this particular area, although genotype 3 HEV strains were found in two boars captured in 2007. Recently, a partial 326-nt ORF1 sequence of a wild boar HEV isolate (JBOAR135-Shiz09) was reported [43]: the boar was captured in 2009 in Shizuoka Prefecture, located in the central part of Honshu Island (Supplementary Figure 1). Notably, the JBOAR135-Shiz09 isolate was only 79.4% identical to our wbJOY_06 within the reported 326-nt sequence, thus suggesting that wbJOY_06 and JBOAR135-Shiz09 are distantly related to each other and may be clustered into two distinct genotypes other than genotypes 1-4. To clarify whether wbJOY_06 segregates into a novel genotype, the full-length genomic sequence of the wbJOY_06 isolate must be determined and will be reported elsewhere [48].

In conclusion, the present study revealed that wild boars in Japan have an overall prevalence of anti-HEV IgG of 8.1% and HEV RNA of 3.3%, that they harbor heterogeneous HEV strains of 3, 4, and unrecognized genotypes, and that wild boars are an important reservoir for HEV in humans, similar to domestic pigs. It is very likely that the consumption of boar meat puts individuals at a high risk of acquiring HEV infection [15, 20, 39, 50], and caution is therefore required in cooking and eating boar meat, although contact with the reservoirs may also have a role in the transmission. Since the presence of HEV strains of previously unidentified genotype(s) was suggested by the findings in this study, further efforts to search for new HEV strains that may be classified into novel genotype(s), not only in wild boars but also in humans and other animals, are therefore warranted.

**Acknowledgments** This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labour and Welfare of Japan. We thank Yoshimoto Seki, Masayuki Nakayama (Ibaraki), Emiko Fukui (Tochigi), Arisa Watanabe, Takako Taketomi, Takahiko Fukuchi (Saitama), Naoki Doi (Kanagawa), Hideki Shinno, Katsuhiko Hotta, Hidemi Honda, Momoko Note (Toyama), Michikazu Takahashi (Fukui), Toyomi Koda, Fumiaki Kawazuma (Nagano), Katsumi Ito, Yoshinori Koga (Gifu), Yasushi Fujiwara (Nara), Hiroto Tanaka (Wakayama), Ayumu Kawamoto, Kazuyoshi Murao (Tottori), Shinji Fujieda, Kimihiro Tanaka (Okayama), Akira Yoshimura, Nobuo Sakuma, Hitoshi Nishimura (Yamaguchi), Takuya Hashimoto, Tatsuhiko Shiraishi, Masashi Bando, Shinji Fujiwara (Tokushima), Hideto Sonoda (Saga), Kazumi Yamazaki (Nagasaki), Kazushi Serikawa, Susumu Hijioka, Makoto Nishimura (Kumamoto), Takeshi Sugimoto (Oita), Shuntaro Matsuda, Takahisa Kawano, and Hidehito Shirao (Miyazaki) for supplying serum and/or liver specimens obtained from wild-caught boars.

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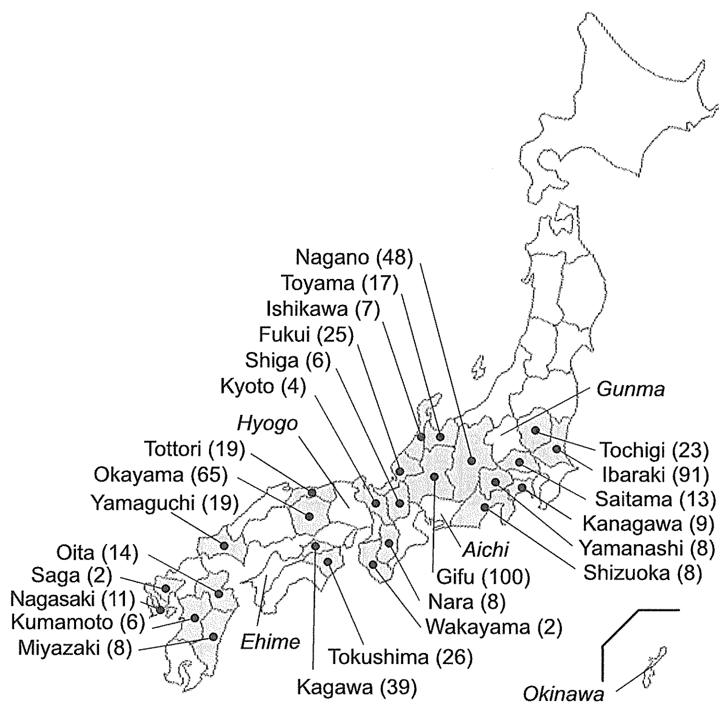


**Supplementary Fig. 1**

A map of Japan showing the locations of the 25 prefectures where the wild boars were captured. The numbers in parentheses indicate the number of wild boars examined in the indicated location. The five prefectures in italics are the prefectures in which HEV-infected boars have been reported [12, 23, 27, 38, 42].

**Supplementary Table 1**

HEV isolates used for phylogenetic analyses in the present study

| Isolate no. | Genotype | Host | Accession no. |
|---|---|---|---|
| 001 | 1 | human | AF051830 |
| 002 | 1 | human | AF076239 |
| 003 | 1 | human | AF185822 |
| 004 | 1 | human | AF444003 |
| 005 | 1 | human | AF459438 |
| 006 | 1 | human | AY204877 |
| 007 | 1 | human | AY230202 |
| 008 | 1 | human | D10330 |
| 009 | 1 | human | D11092 |
| 010 | 1 | human | D11093 |
| 011 | 1 | human | DQ459342 |
| 012 | 1 | human | FJ457024 |
| 013 | 1 | human | L08816 |
| 014 | 1 | human | L25547 |
| 015 | 1 | human | L25595 |
| 016 | 1 | human | M73218 |
| 017 | 1 | human | M94177 |
| 018 | 1 | human | X98292 |
| 019 | 1 | human | X99441 |
| 020 | 2 | human | M74506 |
| 021 | 3 | human | AB074918 |
| 022 | 3 | human | AB074920 |
| 023 | 3 | human | AB089824 |
| 024 | 3 | human | AB091394 |
| 025 | 3 | human | AB189072 |
| 026 | 3 | human | AB189073 |
| 027 | 3 | human | AB189074 |
| 028 | 3 | human | AB189075 |
| 029 | 3 | human | AB246676 |
| 030 | 3 | human | AB248520 |
| 031 | 3 | human | AB291951 |
| 032 | 3 | human | AB291952 |
| 033 | 3 | human | AB291953 |
| 034 | 3 | human | AB291954 |
| 035 | 3 | human | AB291955 |
| 036 | 3 | human | AB291956 |
| 037 | 3 | human | AB291957 |
| 038 | 3 | human | AB291958 |
| 039 | 3 | human | AB291960 |
| 040 | 3 | human | AB291961 |
| 041 | 3 | human | AB291962 |
| 042 | 3 | human | AB291963 |
| 043 | 3 | human | AB301710 |
| 044 | 3 | human | AB369687 |
| 045 | 3 | human | AB369689 |
| 046 | 3 | human | AB369691 |
| 047 | 3 | human | AF060668 |
| 048 | 3 | human | AF060669 |
| 049 | 3 | human | AP003430 |
| 050 | 3 | human | EU495148 |
| 051 | 3 | human | FJ653660 |
| 052 | 3 | human | FJ956757 |
| 053 | 3 | swine | AB073912 |
| 054 | 3 | swine | AB248521 |
| 055 | 3 | swine | AB248522 |
| 056 | 3 | swine | AB290312 |
| 057 | 3 | swine | AB290313 |
| 058 | 3 | swine | AB443623 |
| 059 | 3 | swine | AB481226 |
| 060 | 3 | swine | AB481228 |
| 061 | 3 | swine | AB481229 |
| 062 | 3 | swine | AF082843 |
| 063 | 3 | swine | AF455784 |
| 064 | 3 | swine | AY115488 |
| 065 | 3 | swine | EU360977 |
| 066 | 3 | swine | EU375463 |
| 067 | 3 | swine | EU723512 |
| 068 | 3 | swine | EU723513 |
| 069 | 3 | swine | EU723514 |
| 070 | 3 | swine | EU723515 |
| 071 | 3 | swine | EU723516 |
| 072 | 3 | swine | FJ426403 |
| 073 | 3 | swine | FJ426404 |
| 074 | 3 | swine | FJ527832 |
| 075 | 3 | boar | AB189070 |
| 076 | 3 | boar | FJ705359 |
| 077 | 3 | boar | FJ998008 |
| 078 | 3 | boar | FJ998015 |
| 079 | 3 | deer | AB189071 |
| 080 | 3 | mongoose | AB236320 |
| 081 | 3 | rabbit | FJ906895 |
| 082 | 3 | rabbit | FJ906896 |
| 083 | 3 | rabbit | GU937805 |
| 084 | 4 | human | AB074915 |
| 085 | 4 | human | AB074917 |
| 086 | 4 | human | AB080575 |
| 087 | 4 | human | AB091395 |
| 088 | 4 | human | AB097812 |
| 089 | 4 | human | AB099347 |
| 090 | 4 | human | AB108537 |
| 091 | 4 | human | AB161717 |
| 092 | 4 | human | AB161718 |
| 093 | 4 | human | AB161719 |
| 094 | 4 | human | AB193176 |
| 095 | 4 | human | AB193177 |
| 096 | 4 | human | AB193178 |
| 097 | 4 | human | AB197673 |
| 098 | 4 | human | AB197674 |
| 099 | 4 | human | AB200239 |
| 100 | 4 | human | AB220971 |
| 101 | 4 | human | AB220972 |
| 102 | 4 | human | AB220973 |
| 103 | 4 | human | AB220974 |
| 104 | 4 | human | AB220975 |
| 105 | 4 | human | AB220976 |
| 106 | 4 | human | AB220977 |
| 107 | 4 | human | AB220978 |
| 108 | 4 | human | AB220979 |
| 109 | 4 | human | AB253420 |
| 110 | 4 | human | AB291959 |
| 111 | 4 | human | AB291964 |
| 112 | 4 | human | AB291965 |
| 113 | 4 | human | AB291966 |
| 114 | 4 | human | AB291967 |
| 115 | 4 | human | AB291968 |
| 116 | 4 | human | AB369688 |
| 117 | 4 | human | AB369690 |
| 118 | 4 | human | AB521805 |
| 119 | 4 | human | AB521806 |
| 120 | 4 | human | AJ272108 |
| 121 | 4 | human | EF077630 |
| 122 | 4 | human | FJ763142 |
| 123 | 4 | swine | AB097811 |
| 124 | 4 | swine | AB481227 |
| 125 | 4 | swine | AY594199 |
| 126 | 4 | swine | AY723745 |
| 127 | 4 | swine | DQ279091 |
| 128 | 4 | swine | DQ450072 |
| 129 | 4 | swine | EF570133 |
| 130 | 4 | swine | EU366959 |
| 131 | 4 | swine | EU676172 |
| 132 | 4 | swine | FJ610232 |
| 133 | 4 | swine | GU119960 |
| 134 | 4 | swine | GU119961 |
| 135 | 4 | swine | GU188851 |
| 136 | 4 | swine | GU206559 |
| 137 | 4 | swine | GU361892 |

**Supplementary Table 2**
Reported HEV isolates of human origin with the highest identity for each isolate obtained in the present study

| Genotype/ Isolate name | HEV isolates with the highest identity[a] | | | | | |
|---|---|---|---|---|---|---|
| | ORF1 (412 nt) | | | ORF2 (412 nt) | | |
| | Accession no. | Isolate name | Identity (%) | Accession no. | Isolate name | Identity (%) |
| Genotype 3 | | | | | | |
| wbJSG_03 | AB082550 | HE-JA5 | 92.7 | AB082561 | HE-JA5 | 93.7 |
| wbJTS_04 | AB082554 | HE-JA9 | 95.1 | AB434151 | JRC-HE8 | 93.4 |
| wbJYG_05 | AB082551 | HE-JA6 | 94.4 | AB175483 | HE-JHD1979 | 94.7 |
| wbJSO_05-1 | AB369691 | E097-OSA05C | 95.9 | AB288365 | HE-JSB8177 | 96.1 |
| wbJSO_05-2 | AB369691 | E097-OSA05C | 95.9 | AB288365 | HE-JSB8177 | 96.1 |
| wbJSH_05 | AP003430 | JRA1 | 95.4 | AB079763 | HE-JF2 | 99.0 |
| wbJFI_06 | AP003430 | JRA1 | 94.9 | AB079763 | HE-JF2 | 98.3 |
| wbJYN_06-1 | AB091394 | JJT-Kan | 93.0 | AB175484 | HE-JHD1980 | 96.6 |
| wbJYN_06-2 | AB091394 | JJT-Kan | 93.0 | AB175484 | HE-JHD1980 | 96.6 |
| wbJOY_07-1 | AB369691 | E097-OSA05C | 93.4 | AB175483 | HE-JHD1979 | 91.5 |
| wbJOY_07-2 | AB369691 | E097-OSA05C | 93.4 | AB175483 | HE-JHD1979 | 91.5 |
| wbJNR_10 | AB082552 | HE-JA7 | 92.0 | AB074918 | JKN-Sap | 95.1 |
| wbJNN_09 | AB082550 | HE-JA5 | 93.7 | AB434152 | JRC-HE9 | 97.6 |
| wbJFI_09 | AP003430 | JRA1 | 94.9 | AB079763 | HE-JF2 | 98.1 |
| Genotype 4 | | | | | | |
| wbJGF_06-1 | AB265196 | HE-Aichi-C3 | 99.5 | AB265201 | HE-Aichi-C4 | 99.8 |
| wbJGF_06-2 | AB265196 | HE-Aichi-C3 | 99.5 | AB265201 | HE-Aichi-C4 | 99.8 |
| wbJGF_08-1 | AB265194 | HE-Aichi-C1 | 99.8 | AB265198 | HE-Aichi-C1 | 98.8 |
| wbJGF_08-2 | AB265194 | HE-Aichi-C1 | 99.0 | AB265198 | HE-Aichi-C1 | 99.3 |
| Unclassified genotype | | | | | | |
| wbJOY_06 | AB108654 | HE-JA14 | 81.6 | AB291961 | JMH-Osa04C | 82.0 |

[a] Each isolate was compared with reported HEV isolates of human origin whose 412 nt overlapping sequence within ORF1 or ORF2 is retrievable from DDBJ/EMBL/GenBank databases as of December 2010. Of note, all selected isolates listed above were of Japan origin.