動物

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Rapid communication

Hepatitis E virus infection in wild mongooses of Okinawa, Japan: Demonstration of anti-HEV antibodies and a full-genome nucleotide sequence☆

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Abstract

Hepatitis E virus (HEV), a single-strand RNA virus, has been recovered not only from human beings but also from various species of animals. Here we report our results suggesting that mongoose should be added to the list of reservoir animals of HEV. Of 100 mongooses we examined in Okinawa, Japan, 21 were thought to be positive for anti-HEV antibodies, among which one was definitely positive for HEV RNA. Full-genome sequencing of the HEV isolate revealed that it segregates to a unique subgroup within genotype III. Interestingly, this mongoose strain was closely related to a swine isolate previously reported from Okinawa, implicating the possibility of interspecies transmission between these animals.
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Keywords: Hepatitis E virus (HEV); Single-stranded RNA virus; Zoonosis; Interspecies transmission; Mongoose

1. Introduction

Hepatitis E virus (HEV), which causes acute sporadic hepatitis as well as outbreaks of so-called "water-borne hepatitis" in human beings, was isolated first from human beings [1], next from swine in the United States [2], then from rat in Nepal [3], wild boar and deer in Japan [4,5], and more recently from horse in Egypt [6]. In addition, it has been reported that other animals worldwide such as monkey, goat, cow, sheep, cat, and so on have antibodies against HEV even though viral RNA has not yet been recovered. Here, we report for the first time an HEV isolate from mongoose, a cat-like carnivore of *Herpestidae* family.

2. Materials and methods

2.1. Antibody assay

IgG class antibodies against HEV in the mongooses' sera were determined using an in-house enzyme-linked immunosorbent assay (ELISA), with some modifications of the previously reported method [7]. Briefly, the solid phase was a recombinant capsid protein of HEV, which was kindly provided by Dr. Li Tian-Cheng, and the tracer antibodies were horse radish peroxidase-labled anti-cat rabbit IgG (MP

☆ The nucleotide sequence reported in this paper will appear in DDBJ/EMBL/GenBank databases under accession number AB236320

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Biomedicals Inc., Ohio, USA). We used this anti-cat antibodies under the presumption that it could surrogate anti-mongoose antibodies (commercially unavailable) because mongooses and cats belong to the same *Feliodea* superfamily.

2.2. Detection and sequencing of HEV genome

Detection and nucleotide sequencing of the HEV RNA in the mongooses' sera were performed by the methods described previously [8,9]. Briefly, HEV RNA from the nucleic acids extracted from the mongoose serum was reverse-transcribed to cDNA with use of the THERMOSCRIPT RT System (Invitrogen Corporation, California, USA), and PCR amplification of several overlapping regions of the HEV genome was carried out in the presence of PLATINUM Taq DNA Polymerase High Fidelity (Invitrogen). The 5′- and 3′-terminal sequences were amplified with 5′-Full RACE Core Set (TaKaRa Shuzo Co., Ltd., Shiga, Japan) and

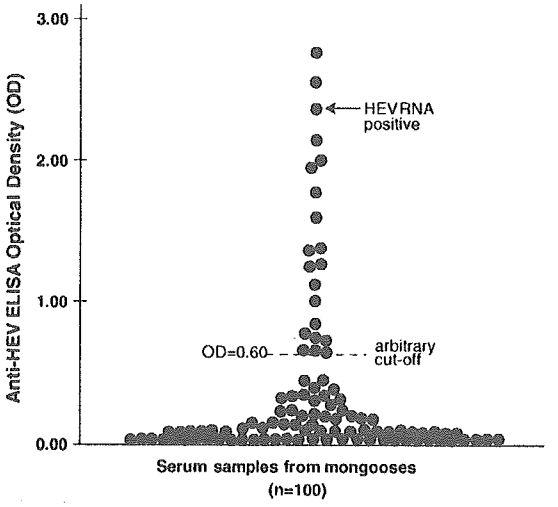

Fig. 1. IgG class antibodies against HEV determined by ELISA.

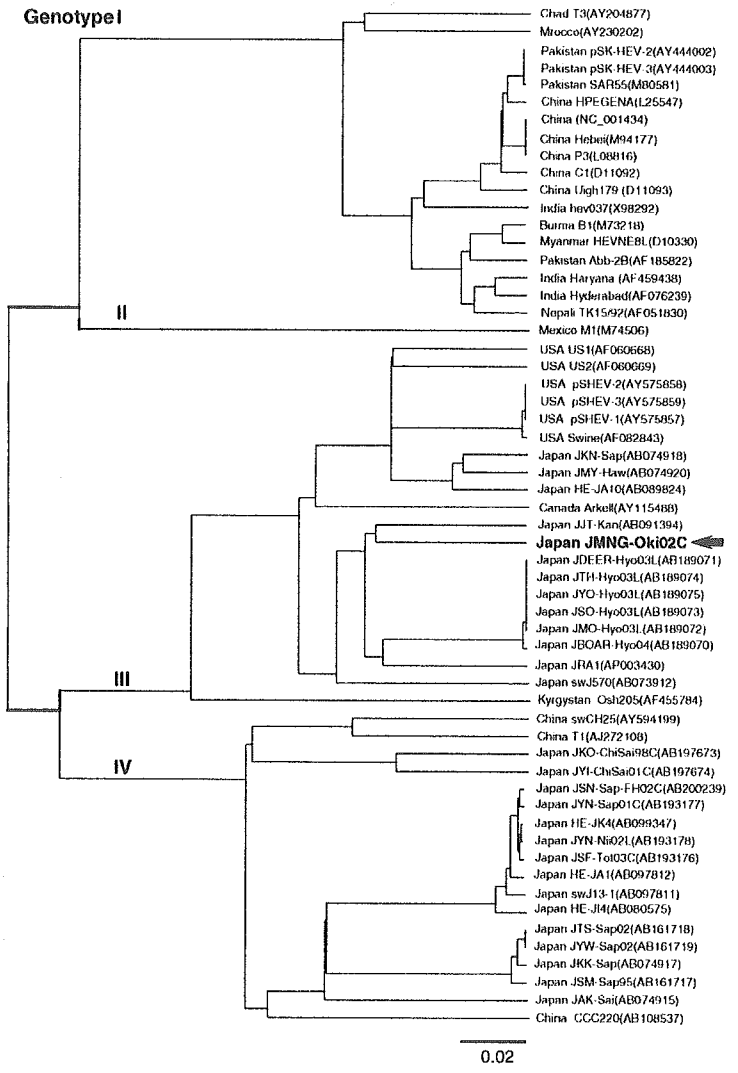

Fig. 2. Phylogenetic tree (UPGMA) based on complete or nearly complete nucleotide sequences of HEV.

3′-RACE System for Rapid Amplification of cDNA Ends (Invitrogen), respectively.

3. Results

3.1. HEV antibodies and RNA in the mongooses' sera

Sera from 100 mongooses captured in 2002 in the mainland of Okinawa prefecture, Japan, were subjected to detection of anti-HEV IgG antibodies and HEV RNA. In an enzyme-linked immunoassay (ELISA) for the antibodies, 21 sera showed optical densities (OD) at greater than 0.600, and were arbitrarily regarded as antibody-positive (Fig. 1). One with the ELISA OD at 2.356 was also positive for HEV RNA by PCR, whose nucleotide sequence was then determined for a partial 412-nt region within ORF2 (isolate name "mnOK1", DDBJ/EMBL/GenBank accession number AB219129), and later for the entire genome (isolate name "JMNG-Oki02C", AB236320).

3.2. The full-genome strain of HEV from mongoose compared to known isolates

The JMNG-Oki02C was comprised of 7236 nucleotides (nt), with the following arrangement of genetic elements from 5′- towards 3′-end: 5′-UTR (nt positions 1–25), ORF1 (nt 26–5137 coding for 1703 amino acids (aa)), ORF2 (nt

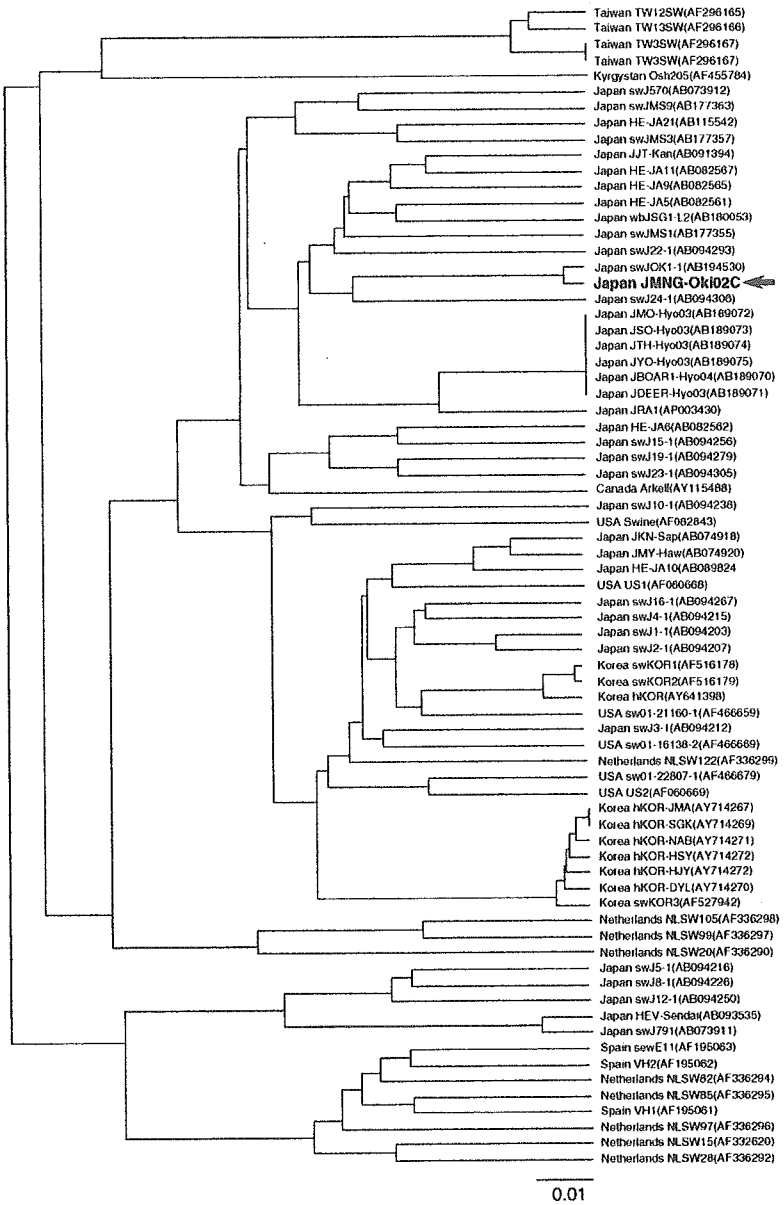

Fig. 3. Phylogenetic tree (UPGMA) based on a partial 412-nt sequence within ORF2. Only isolates of genotype III were included here.

5172–7154 for 660 aa), ORF3 (nt 5134–5502 for 122 aa), 3′-UTR (nt 7155–7226), and a poly-A tail (nt 7227–7236). The residue of nt 6029 was revealed to be Y (both C and T were detected there) in the JMNG-Oki02C isolate (AB236320), whereas we had reported it to be T in the partial sequence mnOK1 (AB219129).

Comparison with full or nearly full-genome HEV isolates so far reported indicated that the mongoose-derived HEV segregates to the genotype III, in particular to a subgroup of those recovered from humans and animals in Japan (Fig. 2). Then when further compared with much wider range of isolates including even those for which only partial sequences had been known, the JMNG-Oki02C sequence showed a strong similarity to one of the swine-derived isolates from Japan (Fig. 3). This swine HEV, swJOK1-1 (AB194530) [10], was an isolate that was obtained from a farm pig in the mainland Okinawa, just the same place where our mongooses were captured.

4. Discussion

Hepatitis E is a zoonosis, supported by direct evidence such as the case where human beings became infected with HEV by eating raw meat (*Sashimi*) of wild deer [4] and by a lot of indirect evidence. Yet we have not known all of the reservoir animals of HEV, unfortunately.

Our present results add to knowledge of the zoonotic aspect of HEV. Since not only antibodies (21%) but also viral RNA were identified, the wild mongoose community we studied in the Okinawa Island is undoubtedly a reservoir, or one of natural hosts, of HEV. Mongoose is a member of the *Herpestidae* family, superfamily of which is *Feliodea*. Of the *Feliodea* animals, only cat has so far been suspected as a reservoir of HEV: presence of antibodies against HEV in pet cat in Japan was once reported [11]. But identification of viral RNA from the *Feliodea* animals has never been successful until our present study.

Another interesting finding in our study was that the mongoose-derived HEV sequence was very homologous to that of a swine isolate (swJOK1-1, AB194530 [10]) which was obtained from a farm pig in the Okinawa Island: nucleotide similarity was 99.5% between JMNG-Oki02C and swJOK1-1 (note: these isolates were analyzed by separate groups of researchers, leaving no chance for laboratory contaminations). Hence, it is possible that an interspecies transmission of HEV might have occurred between pigs and mongooses in Okinawa. Although these animals do not eat each other (mongooses feed on insects, crabs, worms, lizards, and other small creatures), the farm pigs might have been exposed to HEV that was excreted in the feces of mongooses, or vice versa, because mongooses have a cruising radius that is wide enough to intrude the pig farms.

Mongoose was imported into the Okinawa Island in 1910 from India, with an expectation that they might fight and kill the venomous snake *Habu* of Okinawa (because the Indian mongoose was renowned for killing cobras). Although this is merely a speculation, one or some of the imported mongooses might have been infected with HEV at that time, and might transmit it to other will-be-reservoir animals in the Okinawa Island. One of the reasons why we speculate so comes from our unpublished results of molecular clock analyses suggesting that HEV made inroad into Japan around 1900.

Acknowledgments

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Hepatitis E Virus Transmission from Wild Boar Meat

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We investigated a case of hepatitis E acquired after persons ate wild boar meat. Genotype 3 hepatitis E virus (HEV) RNA was detected in both patient serum and wild boar meat. These findings provided direct evidence of zoonotic foodborne transmission of HEV from a wild boar to a human.

Hepatitis E virus (HEV), a causative agent of human hepatitis E, is a single-stranded positive-sense RNA virus recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (1,2). HEV is transmitted primarily by the fecal-oral route through contaminated drinking water. However, recent studies have demonstrated that various animal species have serum antibodies to HEV, suggesting that hepatitis E is a zoonotic disease (3). In Japan, 4 hepatitis E cases have been linked directly to eating raw deer meat (4), and several cases of acute hepatitis E have been epidemiologically linked to eating undercooked pork liver or wild boar meat (5,6). These cases provide convincing evidence of zoonotic food-borne HEV transmission. We report direct evidence of HEV transmission from a wild boar to a human.

The Study

A 57-year-old woman came to Iizuka Hospital on March 12, 2005, with malaise and anorexia. Although she was a healthy hepatitis B virus carrier and negative for serologic markers of hepatitis A and C, testing upon admission showed elevated levels of liver enzymes (alanine aminotranferase 752 IU/L, aspartate aminotransferase 507 IU/L, and γ-glutamyl transpeptidase 225U/L). A serum sample collected on March 16 was positive for both immunoglobulin M (IgM) and IgG antibodies to HEV when tested by an antibody enzyme-linked immunosorbent assay using recombinant viruslike particles (7). This led to the diagnosis of hepatitis E. The hepatitis was typical, acute, and self-limiting, and the patient recovered by the end of March.

The patient's husband traditionally hunted boar for food 3 or 4 times a year, and she had eaten boar meat on 2 occasions. With her husband, she ate the meat as part of a hot pot on December 28, 2004, 11 weeks before her illness, and again, grilled, on January 19, 2005, along with 10 other people (including her husband) 8 weeks before her illness. Disease did not develop in the other 10 people. Except for this wild boar meat, the patient had not eaten meat or liver from other wild animals. Since she had not traveled abroad in the past 30 years, transmission must have occurred in Japan. Two portions of meat from the wild boar (meats 1 and 2) eaten on December 28, 2004, and 1 portion from the other wild boar (meat 3) eaten on January 19, 2005, remained and were frozen.

Juice was obtained from the sliced meat by centrifugation at 10,000 × *g* for 15 min. The supernatant was used for RNA extraction. A nested reverse transcription–polymerase chain reaction (RT-PCR) was conducted to amplify part of open reading frame 2 (ORF2), which corresponds to nucleotides (nt) 5939–6297 of the genotype 1 HEV genome (GenBank D10330), with external sense primer HEV-F1 (5′-TAYCGHAAYCAAGGHTGGCG-3′) and antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-3′). A nested PCR was conducted with internal sense primer HEV-F2 (5′-GGBGTBGCNGAGGAGGAGGC-3′) and internal antisense primer HEV-R1 (5′-CGACGAAATYAATTCTGTCG-3′). This procedure allows amplification of HEV 1, 3, and 4 genotypes. A PCR product of 359 bp including the primer sequences was obtained from meat 3 by nested PCR. However, meats 1 and 2 were negative. HEV RNA was not detected in the patient's serum by the same amplification method. This may have resulted from an extremely small amount of RNA.

New primers for the nested RT-PCR were designed for a region within the 359 base region based on the meat 3 sequences, which corresponded to nt 5983–6243. The first PCR was performed with external sense primer HEV-WB-F1 (5′-ACCTCTGGCCTGGTAATGCT-3′) and antisense primer HEV-WB-R2 (5′-GAGAAGCGTATCAGCAAGGT-3′). The nested PCR was performed with internal sense primer HEV-WB-F2 (5′-TATTCATGGCTCTCCTGTCA-3′) and internal antisense primer HEV-WB-R1 (5′-ACAGTGTCAGAGTAATGCCT-3′). These primers allowed amplification of 281 nt, including the primer sequences from the patient serum collected on March 16, 2005. In contrast, meats 1 and 2 were negative with these new primers.

To further analyze the RNA in the patient serum and meat 3, RNA genomes encoding an entire ORF2 were

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amplified as overlapping segments, nucleotide sequences were determined, and phylogenetic analysis was carried out with avian HEV as an outgroup. Avian HEV is a causative agent of chicken hepatitis-splenomegaly syndrome (8). Two sequences, 1 from the patient (DQ079629) and the other from meat 3 (DQ079630), were classified into genotype 3 (Figure). Only 1 nt difference was observed in the 1,980 nt of the entire ORF2; the nucleotide sequence identity was 99.95%. The difference was not accompanied by any amino acid changes. These data demonstrated that HEV infection was transmitted from the wild boar meat to the patient on January 19, 2005.

Conclusions

Currently, deer, pig, and wild boar are suspected sources of foodborne zoonotic transmission of HEV in Japan, and genotypes 3 and 4 of HEV are believed to be indigenous (4–6,9,10). Direct evidence for transmission of genotype 3 HEV from animals to humans was observed in acute hepatitis in 4 persons who had eaten uncooked deer meat that contained $\approx 10^7$ copies of HEV RNA (4). However, the rare finding of HEV antibody-positive deer in Japan suggest that deer are not the major zoonotic reservoir of HEV in this country (11). In contrast, high antibody-positive rates in domestic pig and wild boar, including HEV genotypes 3 and 4, have been frequently detected, suggesting that persons who eat uncooked meat are at risk for infection with HEV (12,13). This report is the first to provide direct evidence of zoonotic foodborne genotype 3 HEV transmission from wild boar to a human.

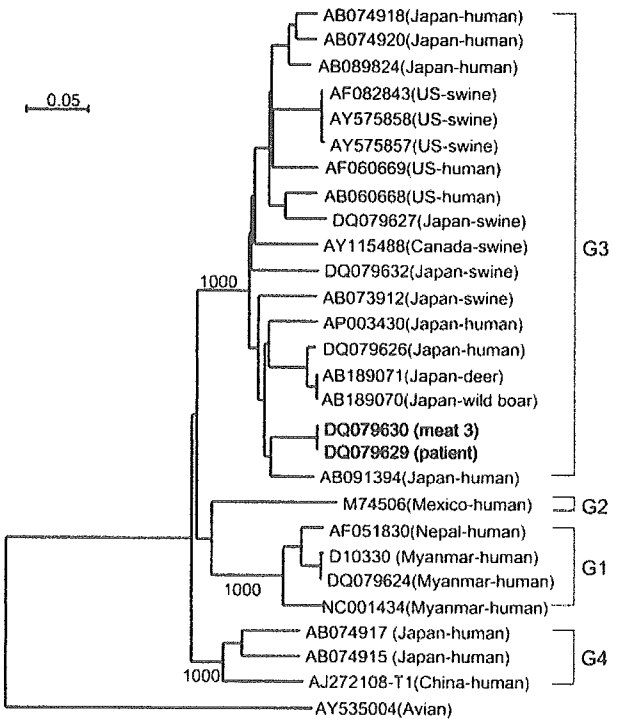

Figure. Phylogenetic tree of hepatitis E virus (HEV) reconstructed with avian HEV as an outgroup. Nucleotide sequences of the entire open reading frame 2 were analyzed by the neighbor-joining method. The bootstrap values correspond to 1,000 replications. The 2 nucleotide sequences characterized in this study are shown in **bold**. The horizontal scale bar at the top left indicates nucleotide substitutions per site.

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Dr Li is a senior researcher at National Institute of Infectious Diseases in Tokyo, Japan. His research focuses on epidemiology, expression of viral proteins, and the three-dimensional structure of hepatitis E virus.

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Short Communication

Analysis of the full-length genome of hepatitis E virus isolates obtained from wild boars in Japan

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Two (2·3 %) of 87 wild-caught boars in Japan had detectable hepatitis E virus (HEV) RNA. The two boar HEV isolates (wbJTS1 and wbJYG1) obtained in the present study and a previously reported isolate (wbJSG1) whose partial sequence had been determined were sequenced over the entire genome. The wbJSG1, wbJTS1 and wbJYG1 isolates comprised 7225 or 7226 nt, excluding the poly(A) tail, and segregated into genotype 3. They differed by 8·5–11·2 % from each other and by 8·6–18·4 % from 17 reported genotype 3 HEV isolates, including one boar isolate, in the full-length sequence. When compared with 191 reported genotype 3 HEV isolates whose partial sequences were known, these three boar isolates were closer to Japanese isolates than to isolates of non-Japanese origin (89·2 ± 2·6 vs 85·9 ± 2·2 %; $P < 0·0001$). A proportion of wild boars in Japan are infected with markedly heterogeneous HEV strains that are indigenous to Japan and may serve as reservoirs of HEV.

Hepatitis E virus (HEV), the causative agent of hepatitis E, is a single-stranded, positive-sense RNA virus without an envelope (Purcell & Emerson, 2001). The genome of HEV is approximately 7·2 kb in size and contains three open reading frames (ORF1–3) (Tam *et al.*, 1991). Extensive genomic diversity has been noted among HEV isolates and HEV sequences have tentatively been classified into four genotypes (genotypes 1–4) (Schlauder & Mushahwar, 2001). The main mode of transmission of HEV in developing countries in Asia, Africa and Latin America is the faecal–oral, water-borne route; large-scale outbreaks via drinking water that was contaminated with faeces containing HEV have been reported. However, in industrialized countries, including Japan, where the water supply and sewage water are treated and disinfected at water-treatment plants, the likelihood of water-borne infection is very low. Recent studies have documented that HEV-associated hepatitis occurs among individuals in industrialized countries with no history of travel to endemic countries (Harrison, 1999; Purcell & Emerson, 2001; Schlauder & Mushahwar, 2001; Smith, 2001) and that HEV is a zoonotic virus (Erker *et al.*, 1999; Hsieh *et al.*, 1999; Meng *et al.*, 1997, 1998; Pina *et al.*, 2000; Wu *et al.*, 2002). It has recently been suggested that zoonotic food-borne transmission of HEV from domestic pigs, wild boars and wild deer to humans plays an important role in the occurrence of cryptic hepatitis E in Japan, where Japanese people have distinctive habits of eating raw fish (sushi or sashimi) and, less frequently, uncooked or under-cooked meat (including the liver and colon/intestine of animals) (Matsuda *et al.*, 2003; Tamada *et al.*, 2004; Tei *et al.*, 2003; Yazaki *et al.*, 2003). The Meng isolate was the first strain of HEV isolated from an animal, namely from an infected pig in the USA in 1997 (Meng *et al.*, 1997). Since then, many swine HEV isolates, which are genetically closely related to strains of human HEV, have been identified in many countries worldwide (Garkavenko *et al.*, 2001; Hsieh *et al.*, 1999; Huang *et al.*, 2002; Meng, 2000, 2003; Nishizawa *et al.*, 2003; Pina *et al.*, 2000; Wang *et al.*, 2002; Wu *et al.*, 2002). In addition, we found a high prevalence of swine anti-HEV antibodies among Japanese pigs of 3–6 months of age

The GenBank/EMBL/DDBJ accession numbers for the complete nucleotide sequences of isolates wbJSG1, wbJTS1 and wbJYG1 reported in this study are AB222182–AB222184, respectively.

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(71 % or 2150/3009) and a high HEV viraemia rate among pigs of 2–4 months of age (11 % or 190/1798) (Takahashi *et al.*, 2003, 2005). Thus, HEV is considered to be enzootic in pigs worldwide. Regarding HEV infection among wild deer, Tei *et al.* (2003) presented data that implicated deer products in the transmission of this disease. However, our previous study revealed that none of 132 wild deer in Japan, including two deer with anti-HEV antibody, had detectable HEV RNA (Sonoda *et al.*, 2004), suggesting that HEV infection via consumption of raw meat or viscera from wild deer occurs very rarely. For HEV from wild boars, although the prevalence of HEV antibody has been reported to be 25 % (15/59) in Australia (Chandler *et al.*, 1999) and 9 % (3/35) in Japan (Sonoda *et al.*, 2004), the genomic characteristics of boar HEV isolates are not fully understood. Therefore, in the present study, we examined the prevalence of HEV infection among wild-caught boars in Japan, and determined and analysed the full-length genomic sequences of three boar HEV strains to clarify the genomic characteristics of the boar HEVs circulating in Japan.

We obtained and analysed paired serum and liver specimens, serum only or liver tissues only from 87 wild boars (*Sus scrofa leucomystax*) that had been captured in the following prefectures (located from north to south in Japan): Ibaraki ($n=15$ boars), Toyama ($n=2$), Nagano ($n=14$), Gifu ($n=1$), Kanagawa ($n=2$), Tottori ($n=19$), Okayama ($n=1$), Wakayama ($n=2$) and Yamaguchi ($n=10$) on mainland Honshu, Kagawa ($n=6$) and Tokushima ($n=14$) on Shikoku Island, and Kumamoto Prefecture ($n=1$) on Kyushu Island, between November 2004 and May 2005. A total of 62 serum samples and 73 liver tissues, including 48 paired serum and liver specimens, were available from the 87 boars: all of the boars in the present study were different from the 41 boars in our previous study (Sonoda *et al.*, 2004). The serum samples were tested for the presence of anti-HEV IgG by an in-house ELISA using purified recombinant ORF2 protein that had been expressed in the pupae of silkworm (Mizuo *et al.*, 2002) as the antigen probe, as described previously (Takahashi *et al.*, 2003). The serum samples and liver tissues were tested for the presence of HEV RNA by RT-PCR using the method described previously with primers targeting the ORF2 region (Mizuo *et al.*, 2002). The RT-PCR assay used has the capability to amplify all four known genotypes of HEV (Mizuo *et al.*, 2002; Takahashi *et al.*, 2003).

The A_{450} value of boar anti-HEV antibodies ranged from 0·002 to 0·645 and one (2 %) of the 62 serum samples had an A_{450} value of $\geqslant$0·300. The sample with an A_{450} value of 0·645 decreased to $<$30 % of the original value after absorption with the same recombinant ORF2 protein that was used as the antigen probe, confirming the specificity of the assay. However, the presence of anti-HEV antibody in three serum samples with an A_{450} value of 0·217, 0·240 or 0·276 was not confirmable by the absorption test. Therefore, only one serum sample from a female boar (wbJTS1) with a body weight of 50 kg that had been caught in Tokushima Prefecture on Shikoku Island on December 9, 2004, was regarded conservatively as being positive for boar anti-HEV IgG in the present study. Among all of the serum and liver specimens from the boars, HEV RNA was detected reproducibly in paired serum and liver specimens obtained from the above-mentioned boar with detectable anti-HEV IgG and in liver specimens obtained from another female boar (wbJYG1) with a body weight of 50 kg that had been caught in Yamaguchi Prefecture on mainland Honshu on February 6, 2005, although a serum sample was not available from the boar. The two HEV-viraemic boars identified in the present study weighed 50 kg, suggesting that the viraemic boars were approximately 2 years of age and that wild boars can acquire *de novo* HEV infection at older ages than domestic pigs, whose HEV RNA is generally detectable between 2 and 4 months of age. The two HEV-infected boars had no clinical manifestations. The HEV sequences amplified from the serum and liver tissue of the wbJTS1 boar were identical in a 412 nt sequence of the ORF2 region.

Taking the results of the present study with those of our previous study (Sonoda *et al.*, 2004), three (2·3 %) of the 128 wild boars captured in 16 prefectures of Japan were viraemic for HEV. To determine the full-length sequence of the HEV isolates (wbJSG1, wbJTS1 and wbJYG1) from the three infected boars, total RNA was extracted from boar liver specimens (100 mg) by using TRIzol reagent (Invitrogen) and the RNA preparation thus obtained was reverse-transcribed and subjected to nested PCR. The central 7 kb sequence of each of the wbJSG1, wbJTS1 and wbJYG1 genomes was divided into seven overlapping sections and amplified: these were nt 43–1270 (1228 nt) (primer sequences excluded), nt 1081–2623 (1543 nt), nt 2605–3127 (523 nt), nt 3106–4700 (1595 nt), nt 4651–5975 (1325 nt), nt 5960–6380 (421 nt) and nt 6342–7199 (858 nt). The extreme 5′-end sequence (nt 1–50) was determined by a modified rapid amplification of cDNA ends (RACE) technique called RNA ligase-mediated RACE (RLM-RACE) with a First Choice RLM-RACE kit (Ambion), as described previously (Okamoto *et al.*, 2001). Amplification of the extreme 3′-end sequence [nt 7101–7225 or 7101–7226 excluding the poly(A) tail] was attempted by the RACE method, according to the method described previously (Okamoto *et al.*, 2000). To avoid contamination during PCR procedures, the guidelines of Kwok & Higuchi (1989) were strictly observed. The amplification products were sequenced on both strands either directly or after cloning into pT7BlueT-Vector (Novagen) and sequence analysis was performed as described previously (Okamoto *et al.*, 2001). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) and bootstrap values were determined on 1000 resamplings of the datasets (Felsenstein, 1985).

The wbJSG1 and wbJYG1 isolates had the same genomic length of 7226 nt, excluding the poly(A) tract at the 3′ terminus, whereas wbJTS1 had a genomic length of 7225 nt, the difference in genomic length being attributed to a deletion of 1 nt in wbJTS1 in the 3′ untranslated region

(UTR). Each of the three isolates possessed three major ORFs, similar to reported human and swine HEV isolates (Meng *et al.*, 1997; Tam *et al.*, 1991; Wang *et al.*, 2000). In each isolate, ORF1, ORF2 and ORF3 had a coding capacity of 1703 aa (nt 26–5134), 660 aa (nt 5172–7151) and 122 aa (nt 5134–5499), respectively. The 5′ UTR of wbJSG1, wbJTS1 and wbJYG1 each comprised 25 nt, and the 3′ UTR sequence of the wbJSG1, wbJTS1 and wbJYG1 genomes consisted of 74 or 75 nt [excluding the poly(A) tail]. The three isolates showed an overall nucleotide sequence identity of 88·8–91·5 %, with the highest identity of 100 % in the 5′ UTR and the lowest identity of 85·3 % in the 3′ UTR; they had nucleotide (amino acid) sequence identities of 88·1–91·2 % (96·7–97·8 %) in ORF1, 90·3–92·3 % (97·6–97·9 %) in ORF2 and 95·6–96·2 % (93·4–95·1 %) in ORF3. Comparison of the wbJSG1, wbJTS1 and wbJYG1 genomes against 52 reported HEV genomes of genotypes 1–4 whose entire or almost entire nucleotide sequences were known revealed that they were closest to JJT-Kan (a genotype 3 human HEV isolate of Japanese origin; see Fig. 1 for GenBank accession no.) and swJ570 (a genotype 3 HEV isolate obtained from a farm pig in Japan) with identities of 89·2–91·4 %, but were only 73·4–75·5 % similar to the prototype HEV isolates of genotypes 1, 2 and 4 (Sar-55, MEX-14 and T1, respectively) in the nucleotide sequence of the full genome. There were no nucleotides or deduced amino acids that were unique to boar HEV strains over the entire genome. The phylogenetic tree was constructed based on the overlapping, almost-complete genomic sequence of 55 HEV isolates obtained from humans, swine, wild boars and wild deer, using an avian HEV as an outgroup; the HEV-like virus infecting chickens shares only 50 % amino acid sequence identity with human and swine strains and is unlikely to infect humans, as it was not experimentally transmissible to rhesus macaques (Huang *et al.*, 2004). The tree confirmed that wbJSG1, wbJTS1 and wbJYG1 belonged

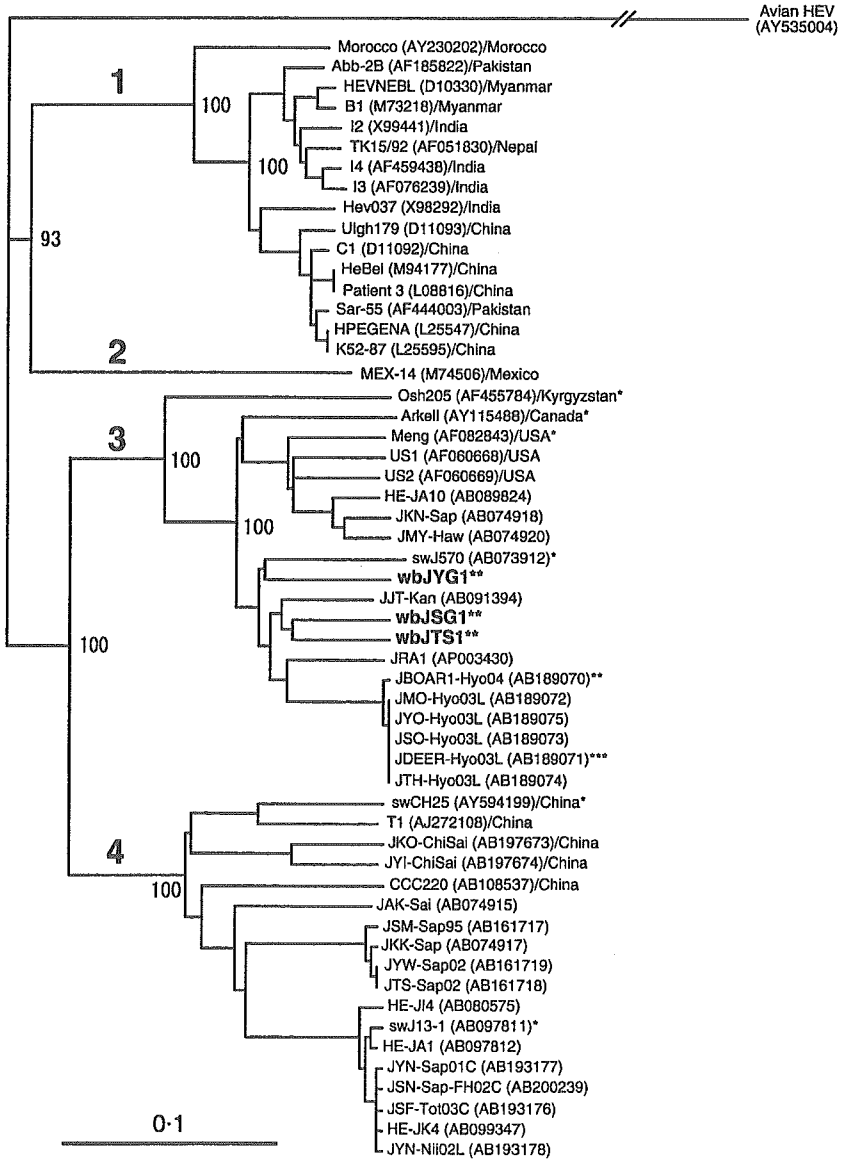

Fig. 1. Phylogenetic tree constructed by the neighbour-joining method based on the nucleotide sequence of 55 HEV isolates obtained from humans, swine, wild boars and wild deer, using an avian HEV isolate (GenBank accession no. AY535004) as an outgroup. As the length of the sequence that has been identified for different isolates varies, the overlapping 7146 nt sequences of 52 reported human, swine, boar and deer HEV isolates and those of the wbJSG1, wbJTS1 and wbJYG1 isolates obtained in the present study (indicated in bold) were compared. GenBank accession numbers are shown in parentheses. After the slash, the name of the country other than Japan where the HEV strain was isolated is shown. Swine HEV isolates, boar HEV isolates and a deer HEV isolate are indicated by asterisks (*, ** and ***, respectively). Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings. Bar, 0·1 substitutions per site.

Table 1. Comparison of three boar HEV isolates of genotype 3 with 191 reported human and swine HEV isolates of the same genotype whose partial sequence is known

Country	Host	Sequence length compared (nt)	Identity (%) [mean ± SD (range)]		
			wbJSG1	wbJTS1	wbJYG1
Japan	Human (n=32)	317/412/824	89·3±2·9 (81·8–93·7)	89·7±2·4 (83·5–93·4)	89·3±2·8 (82·0–94·7)
	Swine (n=103)	412/421/824	89·3±2·9 (80·6–93·4)	89·0±2·2 (83·1–95·1)	88·9±2·3 (81·9–94·2)
Subtotal	n=135	317–824	89·3±2·9 (80·6–93·7)	89·2±2·3 (83·1–95·1)	89·0±2·5 (81·9–94·7)
Non-Japan					
USA	Swine (n=27)	300/301	87·5±1·2 (85·4–90·0)	87·9±1·4 (85·3–91·0)	85·7±0·8 (84·7–87·4)
Argentina	Human (n=2)	371	82·9±1·0 (82·2, 83·6)	84·2±1·3 (83·3, 85·2)	84·5±1·7 (83·3, 85·7)
UK	Human (n=1)	287	81·2	81·5	79·1
Netherlands	Swine (n=11)	476	85·1±2·0 (83·0–89·1)	85·4±1·4 (83·8–88·0)	84·5±1·6 (82·1–87·8)
Austria	Human (n=1)	371	85·2	87·9	84·9
Spain	Human (n=2)	304	81·7±0·2 (81·6, 81·9)	81·9±0·5 (81·6, 82·2)	82·7±1·2 (81·9, 83·6)
	Swine (n=1)	304	82·2	81·6	81·9
Italy	Human (n=1)	371	86·3	86·3	84·1
Greece	Human (n=2)	371	83·8±0·4 (83·6, 84·1)	84·5±0·6 (84·1, 84·9)	81·7±1·9 (80·3, 83·0)
Korea	Swine (n=3)	860	87·8±1·2 (86·4–88·6)	86·9±0·7 (86·0–87·3)	87·8±0·3 (87·4–88·0)
Taiwan	Swine (n=3)	304	85·0±0·8 (84·5–85·9)	84·6±0·8 (84·2–85·5)	85·6±0·8 (85·2–86·5)
Australia	Swine (n=1)	289	87·2	88·2	88·2
New Zealand	Swine (n=1)	705	87·8	88·1	87·4
Subtotal	n=56	287–860	86·2±2·2 (81·2–90·0)	86·5±2·2 (81·5–91·0)	85·1±1·8 (79·1–88·2)
Total	n=191	287–860	88·1±3·0 (80·6–93·7)	88·2±2·6 (81·5–95·1)	87·6±3·0 (79·1–94·7)

to genotype 3 and segregated into a cluster consisting of human and swine HEV isolates that are presumed to be indigenous to Japan (Fig. 1). Among 208 HEV isolates of genotype 3 that were retrievable from GenBank/EBML/DDBJ as of July 2005, the entire or almost-entire sequence of only 17 isolates (8·2 %) obtained in four countries, i.e. USA, Canada, Kyrgyzstan and Japan, had been determined. When compared with the remaining 191 HEV isolates obtained in the 13 countries listed in Table 1, the wbJSG1, wbJTS1 and wbJYG1 genomes obtained in the present study were closer to human and swine HEV isolates of Japanese origin than to those of non-Japanese origin, with identities of 89·2±2·6 vs 85·9±2·2 % (P<0·0001; Mann–Whitney U-test). Of note, wbJSG1 was most closely related to the Japanese human HEV isolate HE-JHD1980 (GenBank accession no. AB175484), although it differed by 6·3 % (412 nt in ORF2); wbJTS1 was closest to the Japanese swine HEV isolates swJHR1-1 (AB194528) and swJOK1-1 (AB194530), with a difference of 4·9 % (412 nt in ORF2); and wbJYG1 was nearest to the Japanese human HEV isolate HE-JHD1979 (AB175483), with a difference of 5·3 % (412 nt in ORF2). The three boar HEV isolates obtained in the present study were most similar to human or swine HEV isolates of Japanese origin, including those that had circulated in the years 1979 or 1980.

Upon comparison with reported HEV sequences obtained from wild boars (Table 2), the wbJSG1, wbJTS1 and wbJYG1 isolates shared nucleotide identities of 88·8–90·2 % with the JBOAR1-Hyo04 isolate of genotype 3 (Takahashi *et al.*, 2004) over the entire genome and nucleotide identities of only 79·6–80·8 % with two boar isolates of genotype 4 in the 412 nt ORF2 sequence. Among the HEV strains recovered from patients who developed hepatitis E after ingestion of uncooked or undercooked meat or liver from wild boars, the wbJSG1, wbJTS1 and wbJYG1 isolates were closest to two HEV isolates of genotype 3 (ENK-NGS03 and EMN-NGS03) that had been recovered from two patients who had ingested grilled meat from wild boars in Nagasaki Prefecture on Kyushu Island (Tamada *et al.*, 2004), with nucleotide identities of 90·2–93·4 % in the 317 nt ORF1 sequence. Furthermore, the wbJSG1, wbJTS1 and wbJYG1 isolates were merely 75·2–75·8 % similar in the full-length sequence to the JSF-Tot03 isolate of genotype 4 that had been recovered from a patient who had eaten uncooked liver from wild boar in Tottori Prefecture on mainland Honshu (Matsuda *et al.*, 2003). In addition, the three boar HEV isolates obtained in the present study shared only 88·9–90·3 % identity with a single HEV isolate from a leftover portion of the deer meat that had been kept frozen to be eaten in the future and those from four patients who had acquired hepatitis E after consumption of raw meat in Hyogo Prefecture on mainland Honshu (Tei *et al.*, 2003). These results suggest that heterogeneous strains of HEV of genotype 3 or 4 are circulating among wild boars in Japan.

In conclusion, although the route(s) of transmission of HEV among wild boars in Japan remains unknown, a proportion of wild boars are infected with markedly heterogeneous HEV strains that are closer to Japanese human and swine HEV strains than to those of non-Japanese origin. To gain a better understanding of hepatitis E as a zoonosis and to prevent zoonotic transmission of HEV to humans, further

Table 2. Comparison of three boar HEV isolates obtained in the present study with seven reported HEV isolates obtained from wild boars or deer or from patients who contracted hepatitis E after ingestion of meat or liver from wild boars

Animal species	Isolate name (genotype)	GenBank accession no.	Sequence length compared (nt)	Identity (%)		
				wbJSG1	wbJTS1	wbJYG1
Wild boar	JBOAR1-Hyo04 (3)	AB189070	7247	89·9	90·2	88·8
	wbOK126 (4)	AB194830	412	79·9	80·6	80·3
	wbOK128 (4)	AB194831	412	79·6	80·8	80·8
	JSF-Tot03 (4)*	AB193176	7251	75·2	75·8	75·5
	ENK-NGS03 (3)†	AY427956	317	90·9	93·4	90·5
	EMN-NGS03 (3)†	AY427957	317	90·2	93·4	90·5
Wild deer	JDEER-Hyo03L (3)	AB189071	7230	90·0	90·3	88·9

*Obtained from a patient who contracted fulminant hepatitis E after ingestion of raw liver from wild boars.
†Obtained from patients who contracted acute hepatitis E after ingestion of barbecued meat from wild boars.

clinical, ecological and virological studies on HEV infection in humans and candidate animals are warranted to clarify how domestic HEV strains circulating in animal species are transmitted to animals and humans.

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Short Communication

Correlation between positivity for immunoglobulin A antibodies and viraemia of swine hepatitis E virus observed among farm pigs in Japan

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To evaluate the usefulness of detection of antibodies to hepatitis E virus (HEV) to screen for viraemic pigs, serum samples obtained from 1425 1–6-month-old pigs in Japan were tested for swine HEV RNA and IgG, IgM and IgA classes of anti-HEV antibody. Fifty-five (5 %) of the 1071 2–5-month-old pigs were positive for swine HEV RNA, but none of 218 1-month-old pigs or 136 6-month-old pigs had detectable HEV RNA. The prevalence of anti-HEV IgG among the viraemic pigs (67 %, 37/55) was similar to that among the non-viraemic pigs (55 %, 757/1370) and the prevalence of anti-HEV IgM among the viraemic pigs and non-viraemic pigs was 7 and 3 %, respectively. However, anti-HEV IgA was detected significantly more frequently among viraemic pigs than among non-viraemic pigs (55 vs 10 %, $P<0{\cdot}0001$). These results suggest that anti-HEV IgA is more useful than anti-HEV IgM to screen for viraemic pigs.

Hepatitis E virus (HEV), the causative agent of hepatitis E, is classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae*. Its genome is a single-stranded, positive-sense RNA of approximately 7·2 kb, with three partially overlapping open reading frames (ORF1, -2 and -3) (Tam *et al.*, 1991; Huang *et al.*, 1992; Wang *et al.*, 2000). Although only one serotype has been recognized, extensive genomic diversity has been noted among HEV isolates, and HEV sequences have been classified into four major genotypes, 1–4 (Schlauder & Mushahwar, 2001). Transmission of HEV in developing countries occurs primarily via the faecal–oral route through contaminated water supplies (Purcell & Emerson, 2001). Recent studies have indicated that zoonosis is involved in the transmission of HEV, especially in industrialized countries (Meng *et al.*, 1997, 1998b, 1999; Erker *et al.*, 1999; Harrison, 1999; Meng, 2000, 2003; Smith, 2001; Tei *et al.*, 2003; Yazaki *et al.*, 2003). Increasing lines of evidence indicate that pigs are animal reservoirs of HEV and that hepatitis E may be transmitted zoonotically from viraemic animals to humans (Meng *et al.*, 1997; Harrison, 1999; Meng, 2000, 2003; Smith, 2001; Nishizawa *et al.*, 2003; Okamoto *et al.*, 2003; Takahashi *et al.*, 2003a). Numerous HEV strains of genotypes 3 and 4 have been isolated from pigs in both developing and industrialized countries (Hsieh *et al.*, 1999; Pina *et al.*, 2000; Garkavenko *et al.*, 2001; van der Poel *et al.*, 2001; Arankalle *et al.*, 2002; Huang *et al.*, 2002a; Pei & Yoo, 2002; Wu *et al.*, 2002; Choi *et al.*, 2003). However, the extent of genomic variability and geographical distribution of swine HEV strains is not fully understood in Japan and there have been little or no data on the prevalence of IgM and IgA antibodies against swine HEV (anti-HEV) among domestic pigs. In the present study, we aimed to understand further the genomic heterogeneity of swine HEV strains throughout Japan and to elucidate whether detection of particular classes of anti-HEV antibodies is useful as a tool to screen for viraemic pigs.

Serum samples were obtained from 1425 pigs (mean age ± SD, 3·5 ± 1·6 months, range 1–6 months) at 92 commercial farms in 20 prefectures including Hokkaido and Okinawa, which are the northernmost and southernmost prefectures of Japan, respectively (see Table 1): there were no overlapping serum samples or swine herds between the previous studies in seven prefectures (Okamoto *et al.*, 2001; Takahashi *et al.*, 2003a, b; Tanaka *et al.*, 2004) and the present study. In each prefecture, serum samples were collected from 8–360 (71·3 ± 89·1) pigs at 1–20 (4·6 ± 5·5) farms in 2001 and 2002 and were kept below −20 °C until testing.

The serum samples obtained from all 1425 pigs were tested for HEV RNA by RT-PCR with the ORF2 primers as

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequence data reported in this paper are AB194476–AB194530.

Supplementary tables and phylogenetic trees are available in JGV Online.

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Table 1. Age-dependent prevalence of HEV RNA in the sera of farm pigs in 20 prefectures in Japan

–, Not tested or not applicable.

Prefecture	Prevalence of HEV RNA (%) among pigs aged:							Names of HEV isolates
	1 month	2 months	3 months	4 months	5 months	6 months	Total	
Hokkaido	0/15	1/17 (6)	11/37 (30)	0/5	0/17	0/11	12/102 (12)	swJHK1-1, 2-1 to 2-5, 3-1 to 3-3, 4-1, 5-1 and 5-2
Aomori	0/16	1/16 (6)	1/16 (6)	1/16 (6)	0/15	0/11	3/90 (3)	swJAO1-1, 1-2 and 2-1
Iwate	0/39	0/25	5/68 (7)	4/24 (17)	0/81	0/21	9/258 (3)	swJIW1-1 to 1-3, 2-1, 3-1, 4-1 and 5-1 to 5-3
Akita	0/65	4/53 (8)	8/76 (11)	3/53 (6)	2/92 (2)	0/21	17/360 (5)	swJAK1-1, 1-2, 2-1, 3-1 to 3-6, 4-1, 5-1, 6-1, 6-2, 7-1 to 7-3 and 8-1
Miyagi	–	–	0/8	0/3	0/85	0/16	0/112	–
Yamagata	0/3	2/3 (67)	0/3	0/3	0/3	0/3	2/18 (11)	swJYM1-1 and 1-2
Fukushima	0/3	0/3	0/3	0/3	0/3	–	0/15	–
Ibaraki	–	0/15	3/15 (20)	0/15	0/10	0/6	3/61 (5)	swJIB1-1 to 1-3
Chiba	0/4	0/2	0/4	0/1	0/1	0/1	0/13	–
Niigata	0/3	1/15 (7)	0/9	0/18	0/8	0/19	1/72 (1)	swJNI1-1
Toyama	0/9	0/3	1/10 (10)	–	0/10	–	1/32 (3)	swJTY1-1
Shizuoka	0/20	–	2/20 (10)	–	0/20	0/1	2/61 (3)	swJSZ1-1 and 2-1
Mie	0/5	–	0/5	–	0/5	–	0/15	–
Tottori	0/3	1/8 (13)	0/3	0/3	0/3	0/6	1/26 (4)	swJTT1-1
Shimane	0/3	0/3	0/3	1/3 (33)	–	0/2	1/14 (7)	swJSM1-1
Hiroshima	0/3	1/3 (33)	0/3	0/3	0/3	0/2	1/17 (6)	swJHR1-1
Kagawa	0/15	0/15	0/15	1/15 (7)	0/15	0/9	1/84 (1)	swJKG1-1
Kochi	0/6	0/6	0/6	0/6	0/6	0/3	0/33	–
Miyazaki	–	0/5	–	0/3	–	–	0/8	–
Okinawa	0/6	0/6	1/6 (17)	0/6	0/6	0/4	1/34 (3)	swJOK1-1
Total	**0/218**	**11/198 (6)**	**32/310 (10)**	**10/180 (6)**	**2/383 (0·5)**	**0/136**	**55/1425 (4)**	

described previously (Mizuo *et al.*, 2002). Although none of the 218 1-month-old pigs or 136 6-month-old pigs had detectable HEV RNA, HEV RNA was detectable in the swine serum samples from 11 (6%) of the 2-month-old pigs, 32 (10%) of the 3-month-old pigs, 10 (6%) of the 4-month-old pigs and two (0·5%) of the 5-month-old pigs (Table 1). HEV RNA was detected in pigs from 31 of the 92 farms tested. According to prefecture, pigs from 14 of the 20 prefectures had detectable HEV RNA; in the remaining six prefectures where no viraemic pigs were detected, the number of pigs aged 2–4 months in this study ranged from only five to 18. Therefore, in total, 55 viraemic pigs were identified in the current study. The swine HEV isolates recovered from the infected pigs were named as shown in Table 1.

The amplification products of ORF2 (412 nt; primer sequences at both ends excluded) of the HEV isolates from the 55 viraemic pigs were sequenced directly on both strands as described previously (Okamoto *et al.*, 2001) and sequence analysis was performed by using Genetyx-Mac version 12.2.6 (Genetyx) and ODEN (version 1.1.1) from the DDBJ (Ina, 1994). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) based on the partial nucleotide sequence of the ORF2 region (412 or 298 nt). The 55 swine HEV isolates obtained from the viraemic pigs were 76·4–100% identical to each other and segregated into two phylogenetic groups (Fig. 1). Among the 55 swine HEV isolates obtained in the present study, 52 (95%) were close to the reported genotype 3 HEV isolates of Japanese and non-Japanese origin, with an identity of 79·9–98·8 (89·1±2·8) and 79·6–96·1 (86·9±2·7)%, respectively, at the nucleotide level, but they differed by 17·2–27·9% from known HEV isolates of the other three genotypes (1, 2 and 4) (see Supplementary Table S1, available in JGV Online). The remaining three isolates were 87·9–93·0 (91·1±2·0) and 83·5–89·3 (86·5±1·2)% similar to reported genotype 4 HEV isolates of Japanese and non-Japanese origin, respectively, but differed by 19·2–26·6% from known HEV isolates of the other three genotypes. These results indicate that 52 and three Japanese swine HEV isolates obtained in the present study are classifiable into genotype 3 and genotype 4, respectively, and that these swine genotype 3 and genotype 4 HEV isolates are closer to known Japanese HEV isolates than to HEV isolates of non-Japanese origin of the respective genotype, as illustrated in the Supplementary Figure (available in JGV Online). In Japan, a total of 212 swine genotype 3 HEV isolates and 13 swine genotype 4 HEV isolates have been identified to date, including those obtained in the present

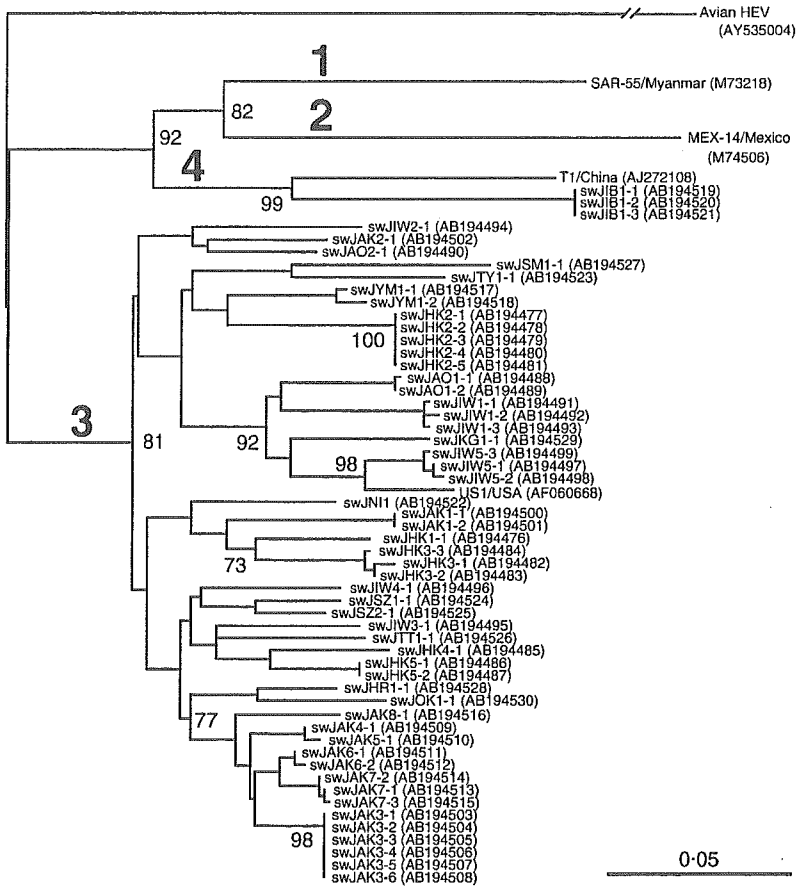

Fig. 1. Phylogenetic tree constructed by the neighbour-joining method based on the partial nucleotide sequence (412 nt; nt 5987–6398 of the HE-JA10 genome, GenBank accession no. AB089824) of the ORF2 region of 59 human and swine HEV isolates, using a chicken HEV isolate (AY535004) as an outgroup. Four prototype human HEV isolates of each of genotypes 1–4, whose entire sequences are known, and the 55 Japanese swine HEV isolates obtained in the present study were included. After the slash, the name of the country other than Japan where the HEV strain was isolated is shown. Bootstrap values of >70% are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings (Felsenstein, 1985).

study. These swine genotype 4 HEV isolates are 0–11·9% different from each other and 10·7–16·5% different from swine genotype 4 HEV isolates reported outside Japan (China, India, Indonesia and Taiwan). More remarkably, Japanese swine HEV isolates of genotype 3 differed by 0–20·1% from each other and by 4·3–20·4% from those of non-Japanese origin (Canada, the Netherlands, Spain, Taiwan and the USA), indicating that Japanese pigs are infected with HEV strains of two distinct genotypes that are markedly heterogeneous.

To detect swine anti-HEV IgG, an in-house ELISA was performed as described previously (Takahashi *et al.*, 2003a), using purified recombinant ORF2 protein of the HE-J1 strain (genotype 4) that had been expressed in the pupae of silkworm (Mizuo *et al.*, 2002), with the modifications described below. The ELISA microplates (Greiner Bio-One) coated with the recombinant ORF2 protein were prepared as described previously (Takahashi *et al.*, 2005). Each sample was added to each well of the microplate at a dilution of 1 : 100 in 10 mM Tris-buffered saline containing 40% Block Ace (Dainippon Pharmaceutical), 0·18% Tween 20 and a mock protein ($A_{280}=0·1$) that had been obtained from the pupae of silkworm infected with non-recombinant baculovirus. As enzyme-labelled antibodies, peroxidase-conjugated rabbit IgG fraction to swine IgG (whole molecule) (MP Biomedicals) was used for the swine anti-HEV IgG assay, peroxidase-labelled goat IgG specific for the pig IgM-μ chain (Bethyl Laboratories) for the swine anti-HEV IgM assay and purified goat IgG against porcine IgA (Serotec) for the swine anti-HEV IgA assay. The A_{450} of each sample was read. To determine the cut-off values in the swine anti-HEV IgG, anti-HEV IgM and anti-HEV IgA assays, 118 control swine serum samples that were exclusively negative for HEV RNA (Takahashi *et al.*, 2003a) were used as a panel. An A_{450} value of 0·274, 0·335 and 0·303 (mean + 6SD) was used as the tentative cut-off value for the swine anti-HEV IgG, IgM and IgA assays, respectively. Test samples with A_{450} values equal to or greater than the cut-off value were considered positive for anti-HEV IgG, anti-HEV IgM or anti-HEV IgA. The specificity of the anti-HEV assays was verified by absorption test with the same recombinant ORF2 protein (50 μg ml^{-1} final concentration for anti-HEV IgG or anti-HEV IgA assay; 150 μg ml^{-1} final concentration for anti-HEV IgM assay) that was used as the antigen probe. Briefly, prior to testing, the serum sample was diluted to 1 : 100, 1 : 300, 1 : 1000 or 1 : 3000 to adjust its A_{450} value to below 1·5. If the A_{450} value of the tested sample decreased by ⩾50% in the anti-HEV IgM assay or by ⩾70% in the anti-HEV IgA or IgG assay after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV.

By using the cut-off value of 0·274, serum samples obtained

from the 1425 pigs in Japan were tested for the presence of swine anti-HEV IgG. The serum samples from 794 pigs (56 %) were positive for swine anti-HEV IgG, with the prevalence by prefecture ranging from 34 to 100 %. The prevalence of swine anti-HEV IgG differed remarkably by age, being 10 % among the 1-month-old pigs, 17 % among the 2-month-old pigs, 67 % among the 3-month-old pigs, 83 % among the 4-month-old pigs, 73 % among the 5-month-old pigs and 74 % among the 6-month-old pigs. When restricted to pigs of 3–6 months of age, swine anti-HEV IgG was detected in 73 % of the pigs (739/1009) (see Supplementary Table S2). By using the cut-off values described above, the serum samples obtained from the 1425 pigs in the present study were further tested for the presence of swine anti-HEV IgM and anti-HEV IgA (Table 2). Swine anti-HEV IgM was detected in the serum samples from 41 pigs (3 % or 41/1425), including four viraemic pigs. In contrast, swine anti-HEV IgA was detected in the serum samples from 169 pigs (12 % or 169/1425), including 30 viraemic pigs. When the age-dependent prevalence of anti-HEV antibodies was compared between pigs that were or were not viraemic, the prevalence of anti-HEV IgM did not differ significantly between the viraemic and non-viraemic pigs in any of the three age groups of 2, 3 and 4 months; the prevalence of anti-HEV IgG also did not differ statistically between the viraemic and non-viraemic pigs in any of the four age groups of 2, 3, 4 and 5 months. Of interest, however, was the fact that anti-HEV IgA was detected significantly more frequently among viraemic pigs than among non-viraemic pigs in the age group of 2 months (36 vs 9 %, $P=0{\cdot}0043$) and in the age group of 3 months (63 vs 20 %, $P<0{\cdot}0001$). Among serum samples obtained from all 1425 pigs, the prevalence of anti-HEV antibodies among viraemic pigs and that among non-viraemic pigs did not differ significantly in the anti-HEV IgG assay (67 vs 55 %), although they did differ significantly in the anti-HEV IgM and anti-HEV IgA assays (7 vs 3 %, $P=0{\cdot}0462$; 55 vs 10 %, $P<0{\cdot}0001$, respectively); however, the proportion of viraemic pigs that were positive for anti-HEV IgM was extremely low at 7 %. Although the prevalence of anti-HEV IgA among viraemic pigs was comparable with that of anti-HEV IgG among viraemic pigs, the accuracy of screening for positivity or negativity of HEV viraemia among pigs of 2–5 months of age based on the anti-HEV antibody status was significantly higher in the anti-HEV IgA assay than in the anti-HEV IgG assay [85 % (909/1071) vs 39 % (417/1071), $P<0{\cdot}0001$].

The reported results of the anti-HEV IgG assay in pigs in various parts of the world indicate that anti-HEV IgG is present among pigs in HEV-endemic countries, such as China, India, Indonesia, Nepal and Thailand, as well as among pigs in non-endemic countries, such as Australia, Canada, Korea, New Zealand, Taiwan and the USA (Clayson *et al.*, 1995; Meng *et al.*, 1997, 1999; Chandler *et al.*, 1999; Hsieh *et al.*, 1999; Garkavenko *et al.*, 2001; Arankalle *et al.*, 2002; Wang *et al.*, 2002; Choi *et al.*, 2003; Wibawa *et al.*, 2004). However, there have been little or no data on the prevalence of swine anti-HEV IgM and anti-HEV IgA among domestic pigs. In the present study, the serum samples obtained from 1425 pigs were tested not only for anti-HEV IgG, but also for anti-HEV IgM and IgA to elucidate whether the ability to detect anti-HEV antibodies is correlated with viraemia among pigs in terms of immunoglobulin classes. Among the 1425 1–6-month-old pigs, anti-HEV IgG was detectable in 37 viraemic pigs

Table 2. Age-dependent prevalence and A_{450} values of anti-HEV IgG, IgM and IgA in the serum samples of farm pigs in relation to HEV viraemia

Pigs of age (HEV RNA)	No. samples tested	Swine anti-HEV IgG			Swine anti-HEV IgM			Swine anti-HEV IgA		
		A_{450} value (mean ± SD)*	No. positive samples (%)	*P* value†	A_{450} value (mean ± SD)*	No. positive samples (%)	*P* value†	A_{450} value (mean ± SD)*	No. positive samples (%)	*P* value†
1 month (−)	218	0·970±0·815	21 (10)		–	0		–	0	
2 months (+)	11	1·966±1·017	4 (36)	0·0824	0·817	1 (9)	0·4564	0·461±0·148	4 (36)	**0·0043**
2 months (−)	187	1·880±0·861	30 (16)		0·493±0·111	8 (4)		0·523±0·234	17 (9)	
3 months (+)	32	2·284±0·679	23 (72)	0·5435	0·569±0·161	2 (6)	0·9609	0·718±0·303	20 (63)	**<0·0001**
3 months (−)	278	1·942±0·811	185 (67)		0·558±0·212	18 (6)		0·626±0·359	55 (20)	
4 months (+)	10	2·211±1·158	9 (90)	0·5605	0·604	1 (10)	0·6637	0·976±0·546	6 (60)	0·0592
4 months (−)	170	1·771±0·832	141 (83)		0·512±0·240	11 (6)		0·647±0·337	53 (31)	
5 months (+)	2	1·915	1 (50)	0·4535	–	0	–	–	0	0·7987
5 months (−)	381	1·336±0·841	280 (73)		–	0		0·625±0·255	12 (3)	
6 months (−)	136	1·124±0·697	100 (74)		–	0		0·610±0·047	2 (1)	
Total (+)	55	2·222±0·824	37 (67)	0·0785	0·640±0·151	4 (7)	**0·0462**	0·735±0·369	30 (55)	**<0·0001**
Total (−)	1370	1·549±0·872	757 (55)		0·530±0·201	37 (3)		0·621±0·326	139 (10)	
Overall total	1425	1·580±0·881	794 (56)		0·541±0·198	41 (3)		0·642±0·335	169 (12)	

*A_{450} values of samples that were positive for anti-HEV antibodies and confirmed by the absorption test (see Methods) are indicated.
†*P* values (χ^2 test) that are significant are indicated in bold type.

(67 %) and 757 non-viraemic pigs (55 %) ($P=0{\cdot}0785$) and anti-HEV IgM was detectable in only four viraemic pigs (7 %) and 37 non-viraemic pigs (3 %) ($P=0{\cdot}0462$); although the latter showed a significant difference, anti-HEV IgM was detected in a very small percentage of the pigs and the result was not considered to be useful. Our results suggest that swine anti-HEV IgG and anti-HEV IgM assays are not useful as tools to screen for viraemic pigs. In support of our findings, it has been reported that anti-HEV IgM appeared earlier than anti-HEV IgG, but was detectable for only 1–2 weeks in domestic pigs that had been infected experimentally with swine or human HEV and that the A_{450} values of anti-HEV IgM were relatively low (below 0·5) (Meng *et al.*, 1997, 1998a). In the current study, the A_{450} value of anti-HEV IgM ranged from 0·058 to 0·817 in the 2-month-old viraemic pigs, from 0·065 to 0·682 in the 3-month-old viraemic pigs and from 0·086 to 0·604 in the 4-month-old viraemic pigs; none of the 5- or 6-month-old pigs were positive for anti-HEV IgM. Evidence of clinical disease or elevation of liver enzymes or bilirubin was not found in pigs that had been infected experimentally with swine or human HEV (Halbur *et al.*, 2001; Meng *et al.*, 1998b). Hence, the lack of clinical disease in infected pigs may be associated with an impaired or reduced immune response of anti-HEV IgM.

Of interest, anti-HEV IgA was detected significantly more frequently among viraemic pigs than among non-viraemic pigs (55 vs 10 %, $P<0{\cdot}0001$). In humans, the IgA anti-HEV test has been utilized as an additional confirmatory test for recent HEV infection (Chau *et al.*, 1993; Tokita *et al.*, 2003). Recently, we found that detection of anti-HEV IgA alone or with anti-HEV IgM is useful for serological diagnosis of hepatitis E with increased specificity and longer duration of positivity than that by RNA detection (Takahashi *et al.*, 2005). IgA anti-HEV was detectable in two out of four patients with subclinical HEV infection in the absence of alanine aminotransferase elevation, who were exclusively negative for anti-HEV IgM (Mitsui *et al.*, 2004), suggesting that detection of anti-HEV IgA is useful for serological diagnosis of acute HEV infection in the absence of anti-HEV IgM.

In conclusion, our present study indicates that HEV is highly prevalent among domestic pigs in swine farms distributed from Hokkaido to Okinawa in Japan and that markedly heterogeneous swine HEV strains of genotypes 3 and 4 are circulating in Japan, some of which are highly similar to HEV strains isolated from patients with domestically acquired hepatitis E in the same geographical region. In addition, the current study suggests that some pigs do not have the ability to generate and maintain a detectable antibody level of swine anti-HEV IgM after HEV infection, and that the anti-HEV IgA assay is more useful than the anti-HEV IgM assay as a tool to screen for viraemic pigs. Previous seroepidemiological studies revealed that anti-HEV IgG antibodies are present in numerous animal species other than pigs, including rodents, chickens, dogs, cats, cows, sheep and goats (Smith, 2001; Huang *et al.*, 2002b, 2004; Usui *et al.*, 2004). However, HEV or HEV-like viruses have not yet been isolated from most of these animal species. Whether detection of anti-HEV IgA in pigs and other animals that may be natural reservoirs of HEV is useful as a tool to screen for viraemia deserves further analysis.

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