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Molecular analysis of hepatitis E virus from farm rabbits in Inner Mongolia, China and its successful propagation in A549 and PLC/PRF/5 cells☆

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ABSTRACT

Rabbit hepatitis E virus (HEV) strains have recently been isolated in several areas of China and in the US and France. However, the host range, distribution and zoonotic potential of these HEV strains remain unknown and their propagation in cultured cells has not yet been reported. A total of 211 4-month-old rabbits raised on a farm in Inner Mongolia were tested for the presence of anti-HEV antibodies and HEV RNA. Overall, 121 rabbits (57.3%) tested positive for anti-HEV antibodies, and 151 (71.6%) had detectable HEV RNA. The 174 HEV strains recovered from these viremic rabbits, including two distinct strains each from 23 rabbits, differed from each other by up to 13.6% in a 412-nucleotide (nt) sequence within ORF2, and were 89.3–95.9% identical to the reported rabbit HEV strains in other provinces of China. Three representative Inner Mongolian strains, one each from three phylogenetic clusters, whose entire genomic sequences were determined, shared 79.6–96.7% identities with reported rabbit HEV strains within the entire or 242- to 1349-nt partial genomic sequence. Rabbit HEV strains recovered from liver tissues of rabbits with a high HEV load propagated efficiently in human cell lines (A549 and PLC/PRF/5 cells), suggesting the potential zoonotic risk of rabbit HEV.

1. Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E worldwide, and the disease is associated with both epidemic and sporadic cases. Hepatitis E is a public health concern in many developing countries in Asia and Africa, where HEV is transmitted by the fecal–oral route because of suboptimal sanitation conditions. Sporadic cases of acute hepatitis E have been increasingly reported in many industrialized countries, including the United States, European countries and Japan, where the transmission is mainly zoonotic (Colson et al., 2010; Dalton et al., 2008; Emerson and Purcell, 2003; Ijaz et al., 2005; Meng, 2010; Mizuo et al., 2002; Okamoto et al., 2003; Purcell and Emerson, 2008). Hepatitis E is typically a self-limiting disease with variable severity, presenting as acute icteric hepatitis with clinical symptoms. However, chronic HEV infection has recently been documented in immunocompromised patients, such as solid-organ transplant recipients and human immunodeficiency virus-infected patients (Dalton et al., 2009; Gerolami et al., 2008; Haagsma et al., 2009; Kamar et al., 2008)

HEV is classified as a member of the genus *Hepevirus* of the family *Hepeviridae* (Meng et al., 2011b). Its genome is a single-strand, positive-sense RNA of approximately 7.2 kilobases (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 (Tam et al., 1991). The 5′ two-thirds of the genome contain ORF1, which encodes non-structural proteins involved in viral replication and viral protein processing. ORF2 occupies the 3′-terminal part of the genome and encodes a 660-amino acid (aa) capsid protein. ORF3 mostly overlaps with ORF2, and encodes a small protein of 113–114 aa that is required for virion egress from cells (Emerson et al., 2010; Yamada et al., 2009). The ORF2 and ORF3 proteins are encoded by a bicistronic subgenomic RNA (Graff et al., 2006).

At least four major genotypes (genotypes 1–4) of HEV that infect humans have been identified (Okamoto, 2007). Genotypes 1 and 2

☆ The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB740220–AB740222 for 3 entire rabbit HEV genomes and AB741083–AB741270 for 188 partial rabbit HEV sequences.

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HEVs are restricted to humans and are often associated with large outbreaks and epidemics in developing countries in Asia and Africa. Genotypes 3 and 4 HEVs have been isolated not only from humans, but also from other animal species, including pigs, wild boars, deer, and mongooses (Meng, 2003; Nakamura et al., 2006; Sato et al., 2011; Takahashi et al., 2004), and are capable of causing zoonotic disease (Li et al., 2005; Tei et al., 2003; Yazaki et al., 2003). Recently, additional putative new genotypes of HEV were identified in wild rats in Germany, USA, and Vietnam (Johne et al., 2010; Li et al., 2011; Purcell et al., 2011), and wild boars in Japan (Takahashi et al., 2010a, 2011). Avian HEV from chickens is phylogenetically distinct from mammalian hepeviruses, and likely represents a new genus within the family *Hepeviridae* (Meng, 2011a).

A cluster of HEV strains that is similar to genotype 3 HEV were recently identified from farm rabbits in China (Geng et al., 2011a,b; Ma et al., 2010; Zhao et al., 2009), the United States (Cossaboom et al., 2011) and France (Izopet et al., 2012). However, the details, including the pathogenesis, geographical distribution, and effect on public health, remain unknown. The objectives of the present study were to investigate whether farm rabbits in Inner Mongolia, China, where rabbit HEV has not yet been identified, are infected with rabbit HEV, and to examine the extent of genetic heterogeneity among rabbit HEV strains in Inner Mongolia, comparing them with those from the other areas of China and the Unites States and France. We also attempted to cultivate rabbit HEV in A549 and PLC/PRF/5 cells, both of which are known to support the efficient replication of various HEV strains recovered from humans, pigs, and wild boars (Takahashi et al., 2010b, 2012; Tanaka et al., 2007). This is the first report demonstrating successful propagation of rabbit HEV in cultured cells.

2. Materials and methods

2.1. Serum and liver samples from rabbits

Serum samples were obtained from 66 Rex rabbits (*Oryctolagus cuniculus*) raised on three farms (22 rabbits each from Central, West, and East Lombok) on Lombok Island of Indonesia in April 2009. A total of 211 serum samples were obtained from 4-month-old Rex rabbits (nos. 1–211) raised on a farm located in the western area of Inner Mongolia, China (Fig. 1) in June 2011. Serum and liver specimens were also collected from 20 additional rabbits (nos. 212–231: 10 aged 3 months and 10 aged 4 months) at the same farm in February 2012. All serum and liver samples were stored at −80 °C until testing.

2.2. ELISA for detecting rabbit anti-HEV IgG

To detect anti-HEV IgG in the serum samples from rabbits, 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 (Mizuo et al., 2002), with slight modifications of the described ELISA method used for the detection of swine anti-HEV IgG (Takahashi et al., 2005). 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% BlockAce (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, a peroxidase-conjugated goat IgG fraction to rabbit IgG Fc (MP Biomedicals, LLC-Cappel, Solon, OH) was used for the rabbit anti-HEV IgG assay. The OD of each sample was read at 450 nm.

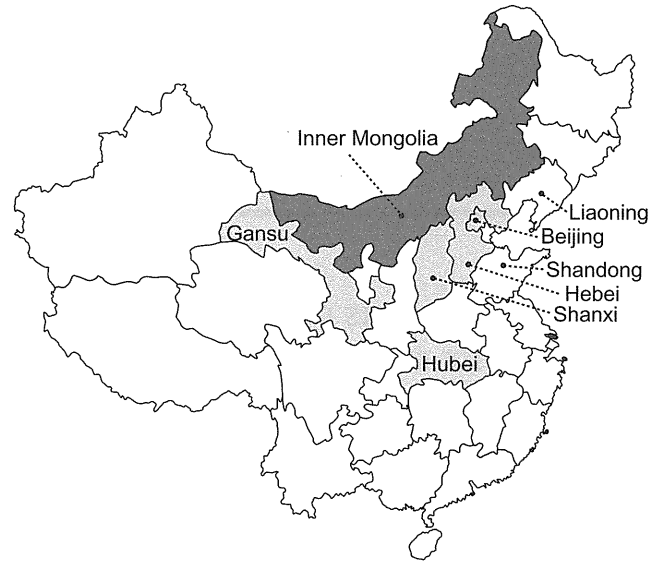

Fig. 1. A map of China showing the locations where the rabbit HEV strains were identified in the present study (Inner Mongolia) and have been identified in the previous studies (shaded areas). The presence of rabbit HEV has been reported in the shaded areas, including Gansu, Hebei, Hubei, and Shanxi Provinces and the capital city, Beijing (Geng et al., 2011b,c; Zhao et al., 2009).

The specificity of the anti-HEV IgG assay was verified by absorption with the same recombinant ORF2 protein (50 μg/ml at the final concentration) that was used as the antigen probe. Briefly, prior to testing, the serum sample was diluted 1:300 to adjust the OD value to <1.5. If the OD value of the tested sample was reduced by ≥70% after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV.

2.3. Qualitative and quantitative detection of HEV RNA

Nested reverse transcription (RT)-polymerase chain reaction (PCR) was performed for the detection of HEV RNA. Total RNA was extracted from 10 to 100 μl of each serum sample or culture supernatant using the TRIzol LS Reagent (Invitrogen, Tokyo, Japan), or 10 to 100 μl of 10% (w/v) liver homogenate using the TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. The extracted RNA was reverse-transcribed with SuperScript II (Invitrogen), and the 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 the method described previously (Mizuo et al., 2002). The size of the amplification product of the first-round PCR was 506 base pairs (bp), and that of the amplification product of the second-round PCR was 457 bp. The RT-PCR assay was performed in duplicate, and its reproducibility was confirmed. The specificity of the RT-PCR assay was verified by a sequence analysis as described below. The sensitivity of the RT-PCR assay was assessed as described previously (Mizuo et al., 2002; Takahashi et al., 2003).

HEV RNA was quantitated by real-time RT-PCR according to a previously described method (Tanaka et al., 2007) with slight modifications, using a culture supernatant containing a known amount of HEV progeny (1.2×10^7 copies/ml) as a standard (Takahashi et al., 2012). In brief, total RNA was extracted from 2 to 100 μl of serum sample, liver homogenate, or culture medium using TRIzol-LS or TRIzol Reagent (Invitrogen) and was subjected to real-time RT-PCR with a QuantiTect Probe RT-PCR Kit (Qiagen, Tokyo, Japan), using 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 oligonucleotide with a 5′-reporter dye (6-carboxyfluorescein, FAM) and a 3′-quencher dye (6-carboxytetramethyl-rhodamine, TAMRA) (5′-FAM-TGA TTC TCA GCC CTT CGC-TAMRA-3′) on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Tokyo, Japan). The thermal cycler conditions were 50 °C for 30 min during stage 1, 95 °C for 15 min during stage 2, and 50 cycles of 94 °C for 15 s and 56 °C for 30 s during stage 3. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate, and the mean value was adopted.

To avoid contamination during these procedures, the guidelines of Kwok and Higuchi (1989) were strictly followed.

2.4. Amplification of the full-length HEV genome

Total RNA was extracted from 200 μl each of three serum samples, and was subjected to cDNA synthesis followed by nested PCR of 5–9 overlapping regions in the central portion of the HEV genome, excluding the extreme 5′- and 3′-terminal regions, using enzymes [KOD-plus (Toyobo, Osaka, Japan), *LA Taq* DNA polymerase (TaKaRa Bio, Shiga, Japan) or *Ex Taq* DNA polymerase (TaKaRa Bio)] and primers whose sequences were derived from well-conserved areas across four major genotypes, as well as those obtained during the amplification procedure: the amplified regions (primers at both ends excluded) are illustrated in Fig. 2. The extreme 5′-end sequence (nt 1–49) 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 (Okamoto et al., 2001). The amplification of the extreme 3′-end sequence [nt 7158–7281 (124 nt) for rbIM199 and rbIM004 and nt 7161–7284 (124 nt) for rbIM022, excluding the poly(A) tail] was accomplished using the RACE method, according to the method described previously (Okamoto et al., 2001).

2.5. Sequence analysis of PCR products

The amplification products were purified using a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) and then were sequenced directly on both strands employing an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). When amplification products showed an ambiguous sequence, they were inserted into the pT7BlueT-Vector (Novagen, Tokyo, Japan), and five independent clones of each were sequenced. The extreme 5′- and 3′-end sequences were determined based on the consensus sequence of at least five clones, respectively. The sequence analysis was performed using the Genetyx software program version 11.0.4 (Genetyx Corp., Tokyo, Japan), and multiple alignments were generated by the CLUSTAL W software program version 2.1 (Thompson et al., 1994). Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987), with the Kimura two-parameter correction model and 1000 replicates of bootstrap re-sampling as implemented in the MEGA 5 software program (version 5.0.5) (Tamura et al., 2011).

2.6. Preparation of inocula for cell culture

Serum samples with a high HEV load were filtered through a microfilter with a pore size of 0.22 μm (Millex-GV; Millipore Corp., Bedford, MA) and were used as an inoculum after a two-fold dilution with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} [PBS(−)] containing 0.2% (w/v) bovine serum albumin (BSA: Sigma–Aldrich Japan, Tokyo, Japan) and were also subsequently subjected to quantification of the HEV RNA. Liver tissues (200 mg) were minced with a razor blade and homogenized with an Eppendorf micropestle (Eppendorf Japan, Tokyo, Japan) in the presence of 1.8 ml of PBS(−), and were clarified by centrifugation in a high-speed micro refrigerated centrifuge (Tomy Seiko, Tokyo, Japan) at 7900 × *g* at 4 °C for 10 min, to obtain a clear supernatant. The 10% (w/v) homogenates filtered through a microfilter with a pore size of 0.8 μm, 0.45 μm, and 0.22 μm (Millex-GV), respectively, were used as an inoculum. Culture medium containing the HEV progeny was also used as the inoculum for supernatant passage after filtration through a microfilter with a pore size of 0.22 μm.

2.7. Cell culture and virus inoculation

A lung cancer cell line (A549, No. RCB0098, RIKEN BRC Cell Bank, Tsukuba, Japan) or a hepatocarcinoma cell line (PLC/PRF/5, ATCC No. CRL-8024, American Type Culture Collection, Manassas, VA) was grown in Dulbecco's modified Eagle medium (DMEM; GIBCO Cat. No. 12800-058), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS: HANA-NESCO BIO, Tokyo, Japan), 100 U/ml of penicillin G, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin B (growth medium) at 37 °C in a humidified 5% CO_2 atmosphere, as described previously (Tanaka et al., 2007). For virus inoculation, cells (3×10^5 cells) in 2.0 ml of medium were added to each well (diameter of 3.5 cm) of a six-well microplate (IWAKI, Tsukuba, Japan) 2 or 3 days before virus infection. Monolayers of cultured cells in the six-well microplates were washed three times with 1 ml of PBS(−), and 0.2 ml of serum sample, homogenated liver suspension, or culture supernatant that had been prepared as described above, was inoculated into each well. One hour after inoculation at room temperature, the solution was removed, and 2 ml of maintenance medium was added. The maintenance medium used for virus culturing consisted of 50% DMEM and 50% medium 199 (GIBCO Cat. No. 31100-027) containing 2% (v/v) heat-inactivated FBS and 30 mM $MgCl_2$ (final concentrations), the other supplements were the same as were contained in the growth medium. The culture was done at 35.5 °C in a humidified 5% CO_2 atmosphere.

On the day following inoculation, the inoculated cells were washed 5 times with 1 ml of PBS(−) and then 2 ml of maintenance medium was added. Then, every other day, one-half (1 ml) of the culture medium was replaced with fresh maintenance medium, and the collected media were stored at −80 °C until virus titrations were performed. In this study, triplicate sets of inocula were inoculated in parallel onto the cultivated cells in a six-well plate. The HEV load was determined for all or selected series of culture supernatants from the inoculated wells as described above. The infected tissue culture microplates were examined daily under an inverted microscope to examine the specific cytopathic effect (CPE).

3. Results

3.1. The prevalence of anti-HEV antibodies and HEV RNA among farm rabbits

In the anti-HEV IgG assay of serum samples obtained from 66 rabbits in Lombok, Indonesia, the OD values ranged from 0.013 to 2.099 (Fig. 3A), although the 66 serum samples were negative for HEV RNA. Seven samples with an OD value of ≥0.527 were confirmed to be positive for anti-HEV IgG by an absorption test with the recombinant ORF2 protein, while six other samples with an OD value of 0.266–0.333 did not show a significant reduction of the OD value after absorption and were considered to be antibody-negative. Using the 59 serum samples with an OD value of 0.013–0.333 that appeared to be negative for anti-HEV IgG as a panel, the OD value of 0.522, which was calculated as 5 SD above the mean value (0.109), was used as the tentative cutoff value.

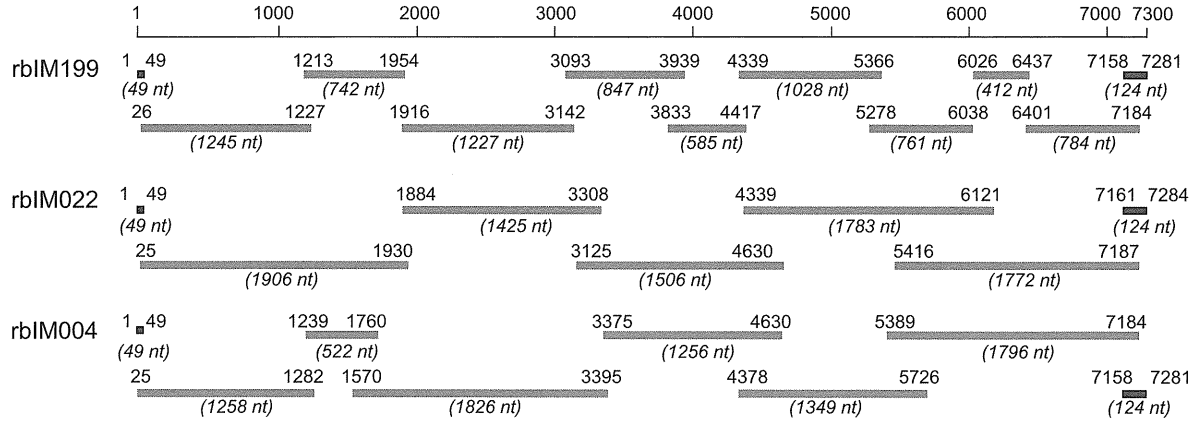

Fig. 2. The strategies used to amplify the three complete rabbit HEV genomic sequences. The line at the top represents the nucleotide position of the rabbit HEV genome. The shaded bars indicate fragments amplified by conventional RT-PCR methods, whose nucleotide sequence was determined by direct sequencing, while closed bars at both ends denote fragments amplified by the RACE technique (see Section 2), whose nucleotide sequence was determined by sequencing at least five independent clones. The numbers over the bars represents nucleotide numbers of the 5′- and 3′-ends of the amplicons whose primer sequences at both ends were excluded. The number in parenthesis under the bar indicates the nucleotide length (nt) of the amplicon (the primer sequences at both ends were excluded).

When serum samples obtained from 211 4-month-old rabbits raised on a farm of Inner Mongolia were tested for the presence of anti-HEV antibodies, 121 samples (57.3%) had OD values that were equal to or greater than the cutoff value, and were considered to be positive for anti-HEV IgG (Fig. 3B). All 211 rabbit serum samples were also tested for the presence of HEV RNA by RT-PCR with nested primers targeting the ORF2 region (ORF2-457 PCR), and 151 samples (71.6%) were found to have detectable HEV RNA.

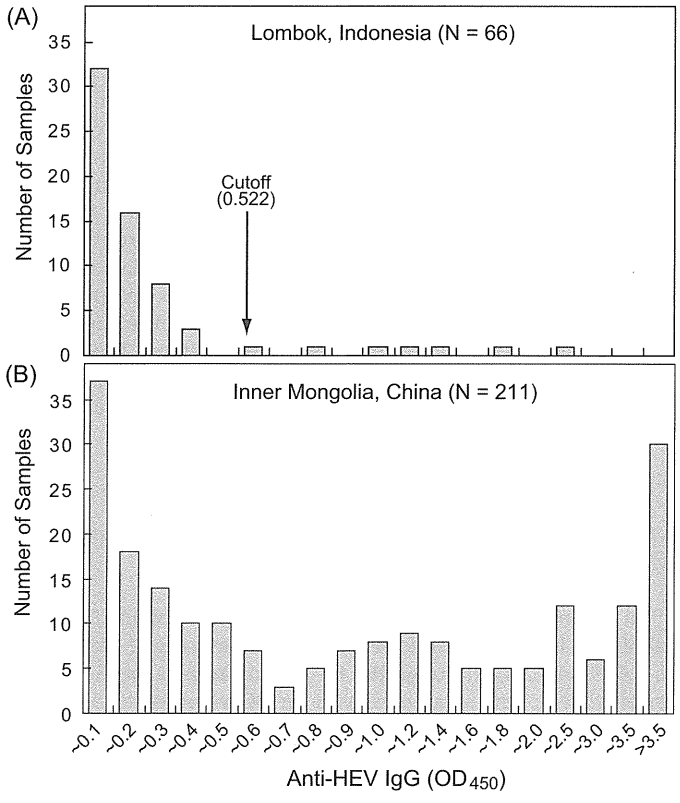

Fig. 3. Distribution of the OD values in the anti-HEV IgG ELISA of serum samples from rabbits in Lombok, Indonesia (A) and Inner Mongolia, China (B). The cutoff value for the anti-HEV IgG test was set at 0.522, which was calculated to be 5 SD above the mean value (0.109), using the 59 serum samples from Indonesia with an OD value of 0.013–0.333, which appeared to be negative for anti-HEV IgG, as a panel.

3.2. Genetic heterogeneity of rabbit HEV strains recovered from one farm in Inner Mongolia

The amplification products of ORF2-457 PCR (412 nt; primer sequences at both ends excluded) from 151 viremic rabbits were sequenced either directly or after molecular cloning, when an ambiguous sequence was obtained by direct sequencing. Each of the 23 samples with ambiguous sequences had two distinct groups of HEV clones, differing from each other by 4.2–11.2%. In total, 174 HEV strains were isolated from the 151 samples of HEV RNA-positive rabbit sera, and their nucleotide sequences were compared. The 174 rabbit HEV strains shared identities of 86.4–100% (93.0 ± 4.0)% within the 412-nt ORF2 sequence, and were most closely related to the reported rabbit HEV sequences (FJ906895, FJ906896, GU937805, JQ768461, JQ013791, and JQ013792), with nucleotide sequence identities of 87.7–95.2 (90.3 ± 1.7)%, 85.0–90.6 (88.2 ± 1.1)%, 88.6–97.1 (92.5 ± 2.3)%, 88.5–96.9 (91.4 ± 2.2)%, 82.9–85.9 (84.4 ± 0.6)%, and 82.5–87.4 (85.2 ± 0.8)%, respectively. The rabbit HEV strains obtained in the present study shared nucleotide sequence identities of 78.5–82.3 (80.5 ± 1.0)% with a prototype HEV strain of genotype 3 [US1 (AF060668)], and only 76.3–80.9 (78.9 ± 0.7)% with a prototype HEV strain of genotype 1 [B1 (M73218)], 73.4–77.0 (74.8 ± 0.6)% with a genotype 2 HEV [MEX-14 (M74506)], and 75.3–78.5 (77.1 ± 0.7)% with a genotype 4 HEV [T1 (AJ272108)]. These results suggest that all 174 Inner Mongolian rabbit HEV strains obtained in the present study are most closely related to reported rabbit HEV strains and are closest to genotype 3 HEV strains among the four known genotypes of HEV strains.

A phylogenetic tree was constructed by the neighbor-joining method based on the 412-nt ORF2 sequences of all 174 HEV strains obtained in the present study, prototype HEV strains of genotypes 1–4, and six rabbit HEV strains from China (FJ906895, FJ906896, GU937805, and JQ768461) and France (JQ013791 and JQ013792), whose entire genomic sequence is known (Fig. 4), using an avian HEV strain (AY535004) as an outgroup. The phylogenetic tree confirmed that all 174 HEV strains are similar to reported rabbit HEV strains and closer to genotype 3 HEV, and suggested that the 174 HEV isolates segregated into three clusters (tentatively designated as Clusters 1, 2, and 3), although the bootstrap values were low (59, 69, and 66%, respectively). In support of this provisional classification, mean identity% within 412-nt ORF2 sequence was 95.4–97.2% within one cluster and 88.4–90.4% between different clusters (Supplementary Table 1). Cluster 1 was predominant,

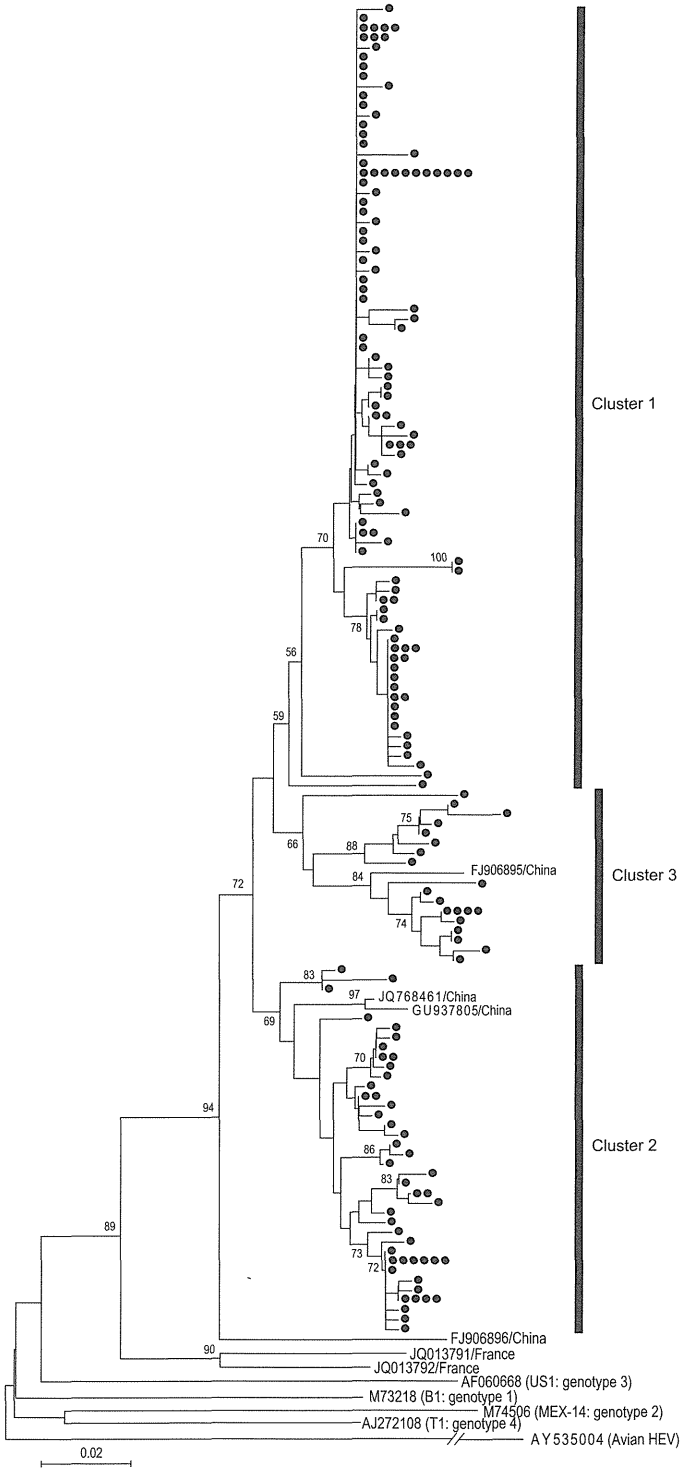

Fig. 4. A phylogenetic tree constructed by the neighbor-joining method based on the 412-nucleotide ORF2 partial sequences of 174 HEV clones obtained in the present study, six representative rabbit HEV strains, and the representative genotypes 1–4 HEV strains, using avian HEV (AY535004) as the outgroup. In addition to the 174 Inner Mongolian rabbit HEV clones, which are indicated only with closed circles for visual clarity, six rabbit HEV strains from China (FJ906895, FJ906896, GU937805, and JQ768461) and France (JQ013791 and JQ013792) whose entire sequence was known, were included for comparison. Three phylogenetic clusters, provisionally designated in this study, are highlighted with vertical bars. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings. The scale bar indicates the nucleotide substitutions per site.

including 106 strains, Cluster 2, represented by GU937805, had 48 strains, and Cluster 3, represented by FJ906895, harbored 20 strains. Of note, all strains from Clusters 1 to 3 were distantly related to FJ906896 (China) and JQ013791 and JQ013792 (France).

To further compare the current HEV with reported rabbit HEV strains whose overlapping 304-nt ORF2 sequences were known, another phylogenetic tree was constructed by the neighbor-joining method based on the 304-nt sequence of prototype genotype 1–4 HEV strains, 53 reported rabbit HEV strains, and 50 selected HEV strains with unique sequence type classifiable into Cluster 1, 2, or 3 obtained in the present study (Fig. 5). Although seven of the 53 reported strains were not classifiable into any of the three clusters, 23 isolates segregated into Cluster 3 (represented by FJ906895), 17 strains into Cluster 1, and 6 strains into Cluster 2 (represented by GU937805): all three clusters were supported by a bootstrap value higher than 70% (82, 72, or 76%, respectively) for Clusters 1, 2 and 3.

3.3. Analysis of the full-length genomes of one representative rabbit HEV strain from Clusters 1 to 3 in Inner Mongolia

The entire genomic sequence was determined for three strains (rbIM199, rbIM022, and rbIM004: highlighted in boldface in Fig. 5), one each from three clusters, including Cluster 1, which possessed 106 strains (60.9%) obtained in the present study, but had no representative isolate whose entire genomic sequence had been determined. The rbIM199 and rbIM004 isolates had a genomic length of 7281 nt, whereas the rbIM022 isolate had a genomic length of 7284 nt, excluding the poly(A) tract at the 3′-terminus. The differences in genomic length were attributed to an insertion of 3 nt (TTT) after nt 5203 in rbIM022 in the junctional region (nt 5195–5217) between the termination codon of ORF1 and the initiation codon of ORF3, similar to two reported rabbit HEV strains (GU937805 and JQ768461).

Each of the three strains obtained in the present study possessed three major ORFs, similar to reported mammalian and avian HEV strains (Meng, 2011a; Tam et al., 1991). In each strain, ORF1, ORF2, and ORF3 encoded 1722 aa (nt 26–5191), 660 aa (nt 5229–7208), and 113 aa (nt 5218–5556), respectively. The 5′- and 3′-untranslated regions of the three strains comprised 25 and 73 nt [excluding the poly(A) tail], respectively.

The rbIM199, rbIM022, and rbIM004 strains shared identities of 85.1–89.7% over the entire genome. The rbIM199 strain was only 80.8–89.6% identical to the reported rabbit HEV strains (FJ906895, FJ906896, GU937805, JQ768461, JQ013791, and JQ013792) over the entire genome, while rbIM022 was most closely related to JQ768461, with the highest identity of 96.0%, and rbIM004 was closest to FJ906895, with the highest identity of 93.6%.

The three rabbit HEV strains obtained in the present study differed from genotype 3 HEV (AF060668) by 21.7–22.1%, while they differed from genotype 1 HEV (M73218) by 28.2–28.6%, from a genotype 2 HEV (M74506) by 28.8–29.2%, and from genotype 4 HEV (AJ272108) by 27.0–27.1%. In addition, the three rabbit HEV strains obtained in this study varied from wild boar HEV [wbJOY_06 (AB602441) and JBOAR135-Shiz09 (AB573435)] by 28.2–28.6%, from rat HEV (GU345042) by 45.5–46.0%, and from avian HEV (AY535004) by 49.5–51.0%.

In order to further characterize the full-length sequences of the rbIM199, rbIM022, and rbIM004 strains obtained in the present study, a phylogenetic tree was constructed based on the entire genomic sequences of nine rabbit HEV strains, including the three strains obtained in the current study, four reported Chinese strains and two recently reported French strains, and genotype 3 HEV strains from humans, domestic swine, wild boars, deer, and mongoose, as well as one representative HEV strain each of genotypes 1, 2, and 4 and two wild boar HEV strains that may be classifiable into novel genotypes, using an avian HEV strain (AY535004) as an

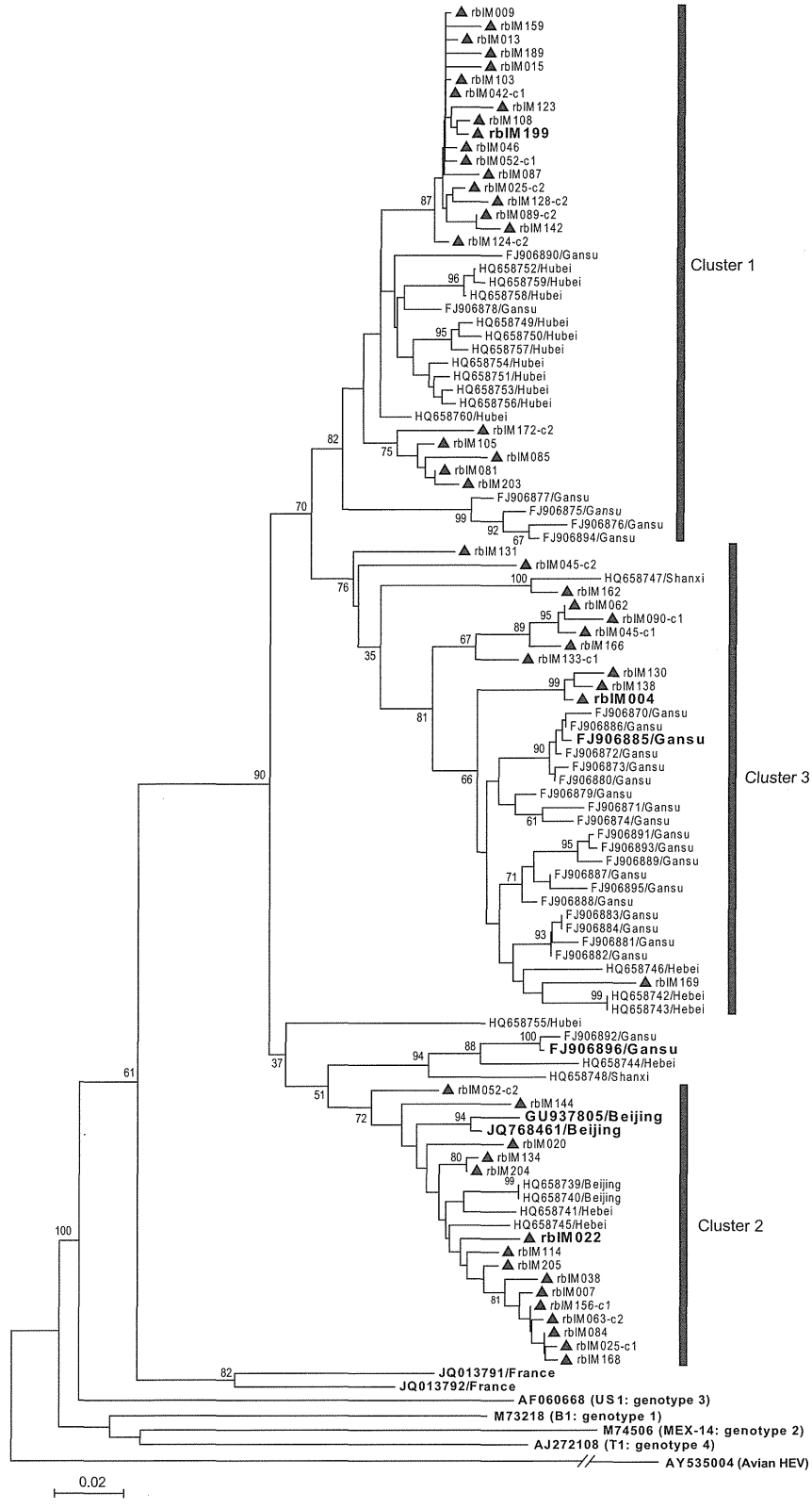

Fig. 5. The neighbor-joining tree of the 304 nt sequence alignment within ORF2 containing 50 selected rabbit HEV strains obtained in the present study, 53 previously reported rabbit HEV strains, and the outgroup strains. In addition to 50 rabbit HEV strains with unique sequence type obtained in the present study (23 from Cluster 1, 15 from Cluster 2, and 12 from Cluster 3) which are indicated with closed triangles, 53 previously reported rabbit HEV strains whose overlapping 304 nt ORF2 sequences were known, and which are shown with accession nos. (followed by the location where it was isolated), were included for comparison. Six reported full-length rabbit HEV genomes (FJ906895, FJ906896, GU937805, JQ768461, JQ013791, and JQ013792) and three Inner Mongolian rabbit HEV strains (rbIM199 of Cluster 1, rbIM022 of Cluster 2, and rbIM004 of Cluster 3) whose entire genomic sequences were determined in the present study, are indicated in boldface type. Three tentative clusters (Clusters 1–3) are depicted with vertical bars. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings. The scale bar indicates the nucleotide substitutions per site.

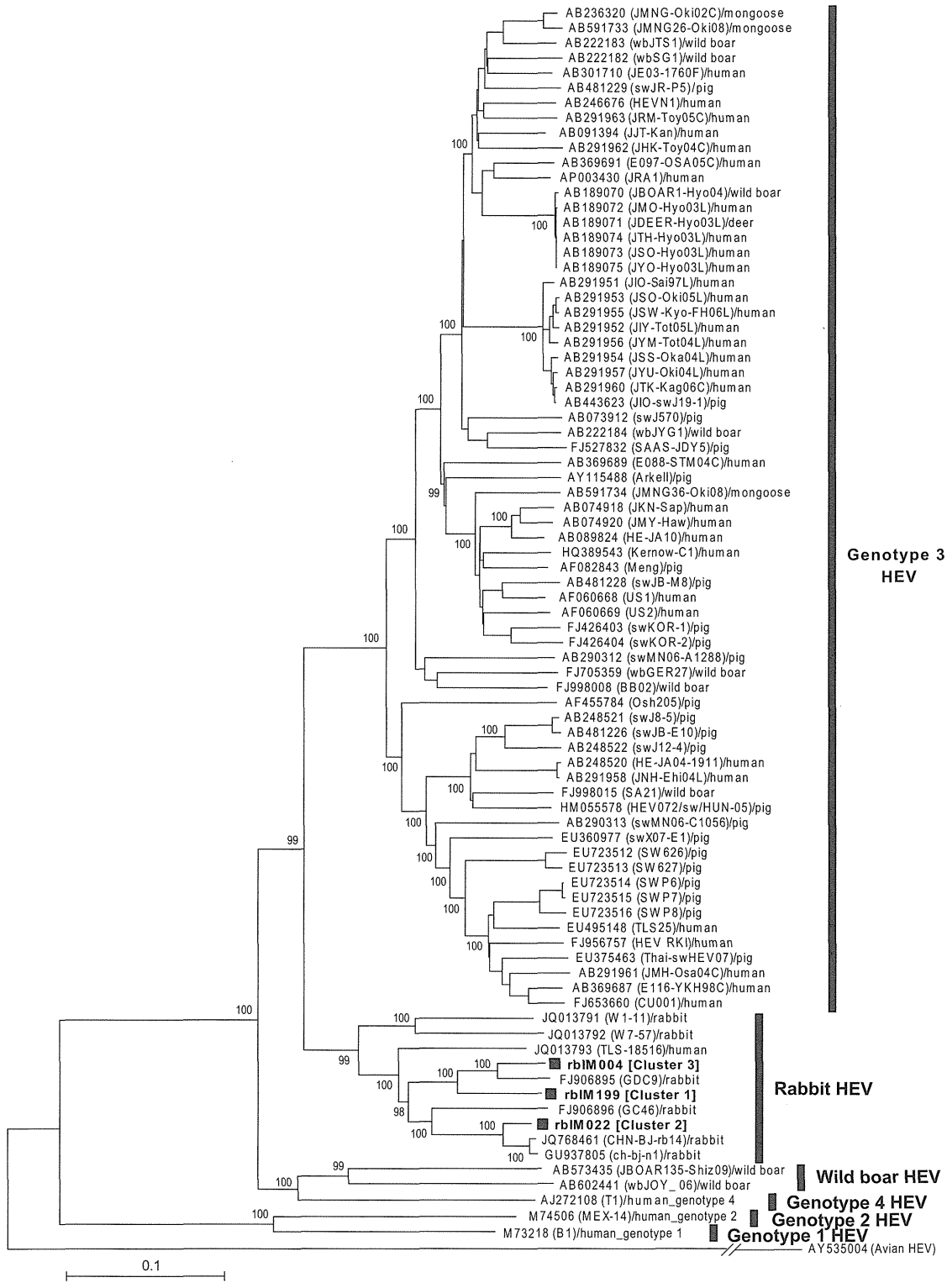

Fig. 6. The neighbor-joining tree of the full-length sequence alignment containing nine rabbit HEV strains, 73 HEV strains from humans, pigs, wild boars, deer, or mongoose, and an outgroup strain. In addition to the three Inner Mongolian rabbit HEV strains whose entire genomic sequences were determined in the present study, and which are indicated in boldface type and highlighted with closed boxes for visual clarity, all six previously reported rabbit HEV strains whose entire sequences were known and all HEV strains of genotype 3, as well as one representative HEV strain each of genotypes 1, 2, and 4, one HEV strain (JQ013793) recovered from a patient with hepatitis E in France that segregates into a cluster of rabbit HEV, and two wild boar HEV strains that may be classifiable into novel genotypes, were included for comparison. The reported strains are indicated with the accession no. followed by the name of the isolate in parenthesis and the name of the infected host from which it was isolated. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings. The scale bar indicates the nucleotide substitutions per site.

Table 1
Comparison of the three rabbit HEV strains of Clusters 1–3 whose entire genomic sequences were determined in this study, with all reported rabbit HEV strains from China, the USA, and France.

Genomic region compared/accession no. of reported rabbit HEV strain	Region[a]	Identity (%)		
		rbIM199 (Cluster 1)[b]	rbIM022 (Cluster 2)[b]	rbIM004 (Cluster 3)[b]
Full genome				
FJ906895	Gansu	89.6	84.3	93.6
FJ906896	Gansu	85.0	87.1	84.4
GU937805	Beijing	85.3	95.7	84.7
JQ768461	Beijing	85.2	96.0	84.8
JQ013791	France	81.0	81.4	87.0
JQ013792	France	80.8	80.8	87.1
ORF1 (1349 nt)				
JQ013789–JQ013790	France	85.7–86.1 (85.9 ± 0.3)	79.6–79.7 (79.7 ± 0.1)	88.8–88.9 (88.9 ± 0.1)
ORF1 (242 nt)				
FJ906853–FJ906869	Gansu	85.1–94.6 (91.0 ± 2.2)	83.9–88.8 (86.9 ± 1.3)	86.0–96.7 (93.9 ± 3.0)
GU186155–GU186159 and JQ768461	Beijing	84.7–86.0 (85.5 ± 0.5)	94.2–95.0 (94.7 ± 0.4)	86.0–86.4 (86.1 ± 0.2)
ORF2 (304 nt)				
FJ906870–FJ906894	Gansu	88.8–96.1 (91.3 ± 1.4)	85.2–89.5 (87.1 ± 1.4)	85.9–95.7 (93.0 ± 2.7)
HQ658741–HQ658746	Hebei	88.2–89.8 (89.3 ± 0.6)	85.9–95.7 (89.8 ± 4.8)	84.2–94.4 (90.2 ± 4.6)
HQ658747–HQ658748	Shanxi	88.8	84.5–90.5 (87.5 ± 2.9)	86.5–90.5 (88.5 ± 2.0)
HQ658749–HQ658760	Hubei	90.1–96.7 (94.8 ± 1.6)	87.5–90.1 (88.3 ± 0.7)	87.2–91.1 (89.9 ± 1.0)
GU086667, GU186160–GU186165, HQ658739–HQ658740	Beijing	89.1–90.5 (89.7 ± 0.5)	94.4–95.7 (95.1 ± 0.5)	87.5–88.8 (88.3 ± 0.5)
ORF2 (765 nt)				
JN383986	USA	90.1	85.4	90.1

[a] For China, the name of the province or city is indicated.
[b] HEV strains whose entire genomic sequence was determined in the present study.

outgroup (Fig. 6). The tree showed that the rbIM199. rbIM022, and rbIM004 strains grouped with the six rabbit HEV strains reported from China (FJ906895, FJ906896, GU937805, and JQ768461) and France (JQ013791 and JQ013792), which are distantly but more closely related to genotype 3 HEV than to any other known HEV genotypes.

As indicated in Table 1, a comparison of the three rabbit HEV sequences of Clusters 1–3 obtained in the present study with reported partial rabbit HEV sequences of 242, 304, 765, or 1349 nt, revealed that rbIM199 (representing Cluster 1) is closely related to some rabbit HEV strains in Gansu and Hubei in China, rbIM022 (representing Cluster 2) to those in Beijing and Hebei, and rbIM004 (representing Cluster 3) to those in Gansu and Hebei, thus suggesting that there is a wide geographical distribution of various rabbit HEV strains in China (Fig. 1).

Although a rabbit HEV strain (USRab-14) has also been identified in the United States (Cossaboom et al., 2011), its partial sequence of 765 nt in the 3′-terminal ORF2 region that is retrievable from public databases overlaps with the 412-nt ORF2 in only the 12-nt sequence. A phylogenetic tree based on the 765-nt sequence indicated that the US rabbit HEV strain is similar to Chinese strains rather than to the French strains within the rabbit HEV cluster (Supplementary Fig. 1)

3.4. Propagation of rabbit HEV in cell culture

Among the 151 HEV RNA-positive serum samples from rabbits, four had a HEV load of 1.3×10^5 to 4.8×10^5 copies/ml, 43 had 1.0×10^4 to 9.5×10^4 copies/ml, and 52 had 1.0×10^3 to 9.8×10^3 copies/ml. Therefore, four serum samples (rbIM001, rbIM043, rbIM119, and rbIM127) with the viral load in the order of 10^5 copies/ml were used as the inocula for culture in A549 cells. The HEV progeny viruses were released into the culture supernatant from day 2–20, differing by inoculum, and then continued to be detectable up through the end of the observation period (day 60) (Fig. 7A). However, the HEV RNA titer in the culture supernatant was low and reached only 10^4 copies/ml, even upon inoculation of the serum sample (rbIM043) with the highest HEV load.

Next, paired serum and liver samples were obtained from 20 additional rabbits (nos. 212–231) raised on the same farm in Inner Mongolia, and their HEV RNA titers were quantitated (Table 2). Five liver homogenate samples obtained from slaughtered rabbits (nos. 214, 223, 226, 227, and 230) with high HEV loads were inoculated onto A549 cells at various HEV RNA titers. The HEV RNA was first detected in the culture medium of A549 cells on the second day post-inoculation (dpi), with a viral load of 6.6×10^1 to 1.6×10^4 copies/ml, and continued to increase thereafter, reaching a HEV load ranging from 7.1×10^4 to 9.6×10^6 copies/ml at 60 dpi. The load of HEV detectable on the initial day of appearance (2 dpi) and the maximum load at 20 dpi differed by the titer of the seed virus in the inoculum. Of note, upon inoculation of a sample (rbIM223L) at the highest load of HEV (6.9×10^7 copies/well), the titer of HEV progeny viruses in the culture supernatant reached 2.2×10^7 copies/ml on day 24 and was maintained at around 10^7 copies/ml up through the observation period of 60 days (Fig. 7B).

PLC/PRF/5 cells also supported successful replication of rabbit HEV upon inoculation of the five rabbit liver homogenate samples with the high HEV loads, although the growth patterns were somewhat more gradual compared with those on A549 cells (Fig. 7C). The rbIM223L grew most efficiently among the five homogenates samples inoculated, and reached 9.5×10^6 copies/ml on day 60.

All HEV progeny viruses released in the culture medium of A549 and PLC/PRF/5 cells, which had been inoculated with the five liver homogenate samples containing high-titer rabbit HEV, were successfully passaged on A549 cells. Fig. 7D depicts the quantitation of HEV RNA in the culture supernatant of A549 cells passaged with three representative progeny viruses derived from A549 cells (rbIM214L_p0, rbIM223L_p0, and rbIM227L_p0).

A phylogenetic tree constructed based on the 412-nt ORF2 sequence showed that each pair of the wild-type virus and its cell culture-generated progeny virus segregated into a cluster within rabbit HEV with a bootstrap value of 100% (data not shown), and shared 99.5–100% nucleotide sequence identity, thereby confirming that various rabbit strains of serum and liver origin were successfully propagated in cultured cells. During these cultivations, no CPE was seen in either A549 or PLC/PRF/5 cells.

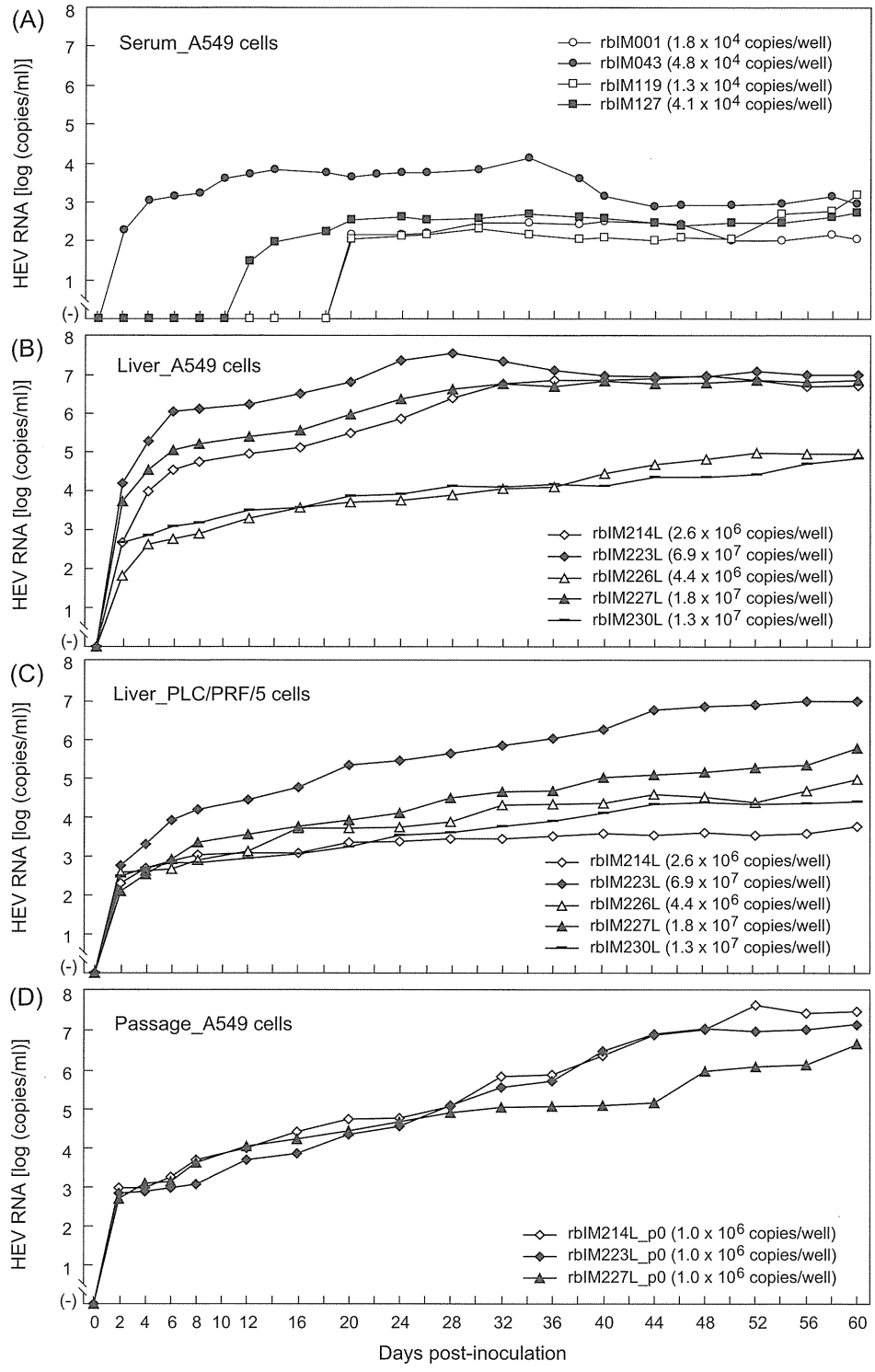

Fig. 7. Quantitation of the HEV RNA in the culture supernatants of A549 cells inoculated with serum samples from viremic rabbits (A), or homogenate samples of rabbit liver tissues (B), or PLC/PRF/5 cells inoculated with homogenate samples of rabbit liver tissues (C), or A549 cells passaged with progeny (Passage 0) in the culture supernatant of A549 cells ((B) at 30 dpi) (D), at the indicated HEV load. For passaging, the harvested culture supernatant at Passage 0 was purified by passing it through a microfilter with a pore size of 0.22 μm (see Section 2 for details) and then inoculated onto fresh A549 cells.

4. Discussion

The present study revealed that farm rabbits in Inner Mongolia, China, are frequently infected with rabbit HEV strains and that polyphyletic strains of rabbit HEV, provisionally classifiable into three clusters differing from each other by 10.3–14.9% over the entire genome, are circulating even within a single farm. We also demonstrated, for the first time, that rabbit HEV can replicate successfully in human cell lines, PLC/PRF/5 and A549 cells, thereby suggesting the potential zoonotic risk of rabbit HEV.

In 2009, Zhao et al. (2009) first reported the presence of a unique strain of HEV, designated rabbit HEV, in farm pigs in Gansu Province, China (Fig. 1). In their study, the overall prevalence of anti-HEV antibodies and HEV RNA was 57.0% (191/335) and 7.5% (25/335),

Table 2
Characteristics of the 20 additional rabbits whose serum and liver specimens were obtained.

ID no.	Age (months)	Sex	Serum			Liver		HEV cluster
			Anti-HEV IgG (OD_{450})	HEV RNA (copies/ml)	Sample name	HEV RNA (copies/ml) in 10% (w/v) homogenate	Sample name	
212	3	M	0.028 (−)	(−)		(−)		
213	3	M	0.041 (−)	<500 (+)	rbIM213S	7.0×10^4 (+)	rbIM213L	2
214	3	F	0.051 (−)	8.3×10^2 (+)	rbIM214S	1.3×10^7 (+)	rbIM214L	1
215	3	F	0.026 (−)	(−)		(−)		
216	3	F	0.012 (−)	(−)		(−)		
217	3	M	0.744 (+)	(−)		<500 (+)	rbIM217L	2
218	3	M	0.025 (−)	(−)		(−)		
219	3	F	0.016 (−)	(−)		(−)		
220	3	F	0.022 (−)	(−)		(−)		
221	3	M	0.378 (−)	(−)		(−)		
222	4	F	0.031 (−)	(−)		(−)		
223	4	F	0.044 (−)	9.1×10^3 (+)	rbIM223S	3.5×10^8 (+)	rbIM223L	2
224	4	F	0.010 (−)	(−)		(−)		
225	4	M	0.032 (−)	<500 (+)	rbIM225S	4.2×10^5 (+)	rbIM225L	2
226	4	M	0.340 (−)	6.0×10^3 (+)	rbIM226S	2.2×10^7 (+)	rbIM226L	3
227	4	F	>3.000 (+)	1.6×10^4 (+)	rbIM227S	9.0×10^7 (+)	rbIM227L	2
228	4	M	1.828 (+)	(−)		<500 (+)	rbIM228L	2
229	4	F	0.025 (−)	(−)		<500 (+)	rbIM229L	2
230	4	M	>3.000 (+)	7.7×10^3 (+)	rbIM230S	6.5×10^7 (+)	rbIM230L	2
231	4	M	0.094 (−)	(−)		(−)		

respectively. In a separate study from Beijing, China, 54.6% (65/119) of the farmed rex rabbits tested positive for anti-HEV antibodies, with 7.0% of fecal samples (8/115) also positive for HEV RNA (Geng et al., 2011b). In additional studies on the prevalence of rabbit HEV in 10 counties in China, 15.4% of the tested rabbits (169/1094), from nine of the 10 counties, were positive for HEV antibodies, with the highest seroprevalence of 53.4% in Wuhan, Hubei Province, while 22 rabbits (2.0%) from Beijing, Hebei, Shanxi, and Hubei had detectable HEV RNA (Fig. 1). The rate of HEV viremia was highest at 11.6% (12/103) in Wuhan, Hubei Province, while no viremic rabbits were identified in 6 of the 10 counties studied (Geng et al., 2011c). Besides China, HEV has been reported in the United States, where farmed rabbits were also found to be naturally infected with rabbit HEV, with the seropositivity for anti-HEV antibodies at 36.5% (31/85) and positivity for HEV RNA in serum at 16.5% (14/85) (Cossaboom et al., 2011). Very recently, Izopet et al. (2012) reported that farmed and wild rabbits in France are naturally infected with HEV (7%, or 14 of 200, bile samples from farm rabbits and 23%, or 47 of 205, liver samples from wild rabbits). As expected, the farm rabbits in Inner Mongolia were also found to be infected with rabbit HEV in the present study. To our surprise, anti-HEV IgG and HEV RNA were detected in approximately 57% and 72%, respectively, of the 211 4-month-old rabbits studied. Among the 151 viremic samples, 95 had detectable anti-HEV IgG, thus representing an ongoing HEV infection accompanied by an early antibody response, and the remaining 56 viremic samples were negative for anti-HEV IgG, most likely representing viremia before the development of an immune response in infected rabbits. The finding that 27 anti-HEV IgG-positive samples were negative for HEV RNA may represent a past HEV infection in the studied rabbits.

The reason for the markedly high prevalence of viremia in the current study remains unknown. However, the high rate of HEV viremia among rabbits on the studied farm was supported by high positivity for HEV RNA in serum (35%) and liver (50%) samples of 20 additional rabbits (Table 2) that were collected 8 months after the first collection of the 211 samples from the same farm. The rabbits on the studied farm were caged in groups of 2–3. Since HEV is transmitted by the fecal–oral route, the virus likely spreads between cage mates, thus increasing the numbers of HEV-infected rabbits. The ages of rabbits, animal housing practices, and hygienic conditions on the rabbit farm may also affect the rate of HEV infection. The specificity of HEV viremia detectable by nested RT-PCR was verified by a sequence analysis. Further confirming the high frequency of HEV infection among the studied rabbits, 23 of the 151 viremic rabbits had two distinct groups of HEV clones.

The rabbit HEV strains obtained in the present study were heterogeneous, as depicted in Fig. 4, and were provisionally classified into three clusters (Clusters 1–3): Cluster 1 (represented by rbIM199) was predominant, accounting for 61% (106/174) of the strains, followed by Cluster 2 (represented by rbIM022) (28%) and Cluster 3 (represented by rbIM004) (11%). In the 20 additional samples collected on the same farm 8 months after the initial sampling, Cluster 2 was observed most frequently and accounted for 80% of the strains from the viremic rabbits, thus suggesting that there are changing dynamics in the virus population even within a single farm, although we cannot rule out the possibility that the smaller number of samples (20 samples in comparison to 211 samples) could also be the reason for the observed shift.

The circulation of rabbit HEV strains with considerable genetic diversity in China has been reported (Geng et al., 2011c), and rabbit HEV strains homologous to those identified in Beijing, Gansu, Hebei, and Hubei were recovered from the rabbits on the farm in the present study (Table 1, Fig. 1). In support of this finding, the farmer told us that rabbits had been purchased from various regions in Mainland China, including Hebei, Liaoning, and Shandong. Due to the low similarity to rabbit HEV found in the United States (85.4–90.1%) and France (79.6–88.9%), it is likely that the Inner Mongolian rabbit HEV strains originated from those circulating in other parts of China.

Phylogenetic analyses indicated that rabbit HEV strains from China, including those obtained in Inner Mongolia in the present study, and from the United States and France form a unique cluster that are distantly related to genotype 3 HEV strains recovered from humans, pigs, wild boars, deer, and mongoose (Meng, 2011a; Okamoto, 2007; Pavio et al., 2010; Sato et al., 2011; Takahashi et al., 2003). The classification of rabbit HEV as a member of a novel genotype or a new subgenotype within genotype 3 remains controversial and unclear. Some researchers have reported that the rabbit HEV strains could be classified into a novel genotype (Geng et al., 2011a,b; Izopet et al., 2012; Zhao et al., 2009), while others have provided a notion that rabbit HEV is likely a

distant member of genotype 3 (Cossaboom et al., 2011; Pavio et al., 2010; Purdy and Khudyakov, 2010). The concept of subdivision of HEV into a subgenotype has evolved from extensive investigation of the genetic variability within four genotypes (1–4) (Lu et al., 2006); however, the proposed classification was based on partial genomic sequences. A unified system of nomenclature for the HEV genotypes/subgenotypes should be determined based on comparative analyses of the complete genome sequences in accordance with many precedents, such as hepatitis C virus (Simmonds et al., 2005) and hepatitis B virus (Kramvis et al., 2008; Okamoto et al., 1988). For this purpose, continued efforts to accumulate sequence data for the complete HEV genomes that may be classifiable into novel genotypes/subgenotypes are needed for future studies. To avoid obfuscation and confusion, the taxonomic classification of the existing rabbit HEV strains and other animal HEV strains recovered from wild boars (Takahashi et al., 2010a, 2011), wild rats (Johne et al., 2010; Li et al., 2011; Purcell et al., 2011), ferrets (Raj et al., 2012), and bats (Drexler et al., 2012) as well as those that might emerge from extended studies should be determined by the International Committee on Taxonomy of Viruses. In the interim, we would like to propose that the HEV strains identified from an animal be designated as HEV with the name of the animal species, e.g., "rabbit HEV".

Acute hepatitis E has been increasingly reported in industrialized countries, including the United States, European countries, and Japan, where the transmission is mainly zoonotic (Dalton et al., 2008; Meng, 2011a; Okamoto et al., 2003). Although infection with genotypes 3 and 4 HEVs has been linked to the consumption of raw or undercooked meats/viscera from pigs, wild boars, and deer (Colson et al., 2010; Tei et al., 2003; Yazaki et al., 2003), the full spectrum of animals that are reservoirs for HEV infection in humans is still unknown. In our previous studies, PLC/PRF/5 cells originating from human hepatocellular carcinoma and A549 cells derived from human lung cancer supported the efficient propagation of HEV strains not only from humans (Okamoto, 2011a,b), but also from pigs and wild boars (Takahashi et al., 2012). These findings encouraged us to cultivate the rabbit HEV strains in PLC/PRF/5 and A549 cells in the present study. Upon inoculation of liver homogenates from rabbits infected with high-titer rabbit HEV, progeny viruses were released into the culture media of A549 and PLC/PRF/5 cells, with the highest HEV load reaching approximately 10^7 copies/ml. The progeny viruses were successfully passaged in A549 cells, indicating the infectivity of the cell culture-generated virions of rabbit HEV. These results suggest the potential transmission of rabbit HEV to humans across species barriers. Recently, Cossaboom et al. (2012) assessed the ability of rabbit HEV to cause cross-species infections in a pig model, and demonstrated that rabbit HEV strains from China and the United States are able to infect pigs when inoculated intravenously, as approximately half of the inoculated pigs developed transient viremia and sporadic fecal shedding. The infection of pigs by rabbit HEV was further verified by transmission of the virus recovered from pig feces to naive rabbits. Izopet et al. (2012) characterized a human HEV strain that is closely related to the rabbit HEV strains that formed a phylogenetic cluster with reported rabbit HEV strains (see Fig. 6 and supplementary Fig. 1), thus supporting the potential of zoonotic transmission from rabbits to humans.

In conclusion, the present study has shown that farm rabbits in Inner Mongolia, China, are infected with rabbit HEV strains, and that heterogeneous strains of rabbit HEV are circulating even within a single farm. We also demonstrated that rabbit HEV strains are able to grow efficiently in human cell lines, PLC/PRF/5 and A549 cells, thus reinforcing the potential zoonotic risk of rabbit HEV. Further studies are needed to show direct evidence of cross-species transmission, as have already been done for deer and wild boars (Li et al., 2005; Tei et al., 2003), and to evaluate the contribution of the rabbit reservoir to the onset of clinical and subclinical HEV infection in humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2012.09.015.

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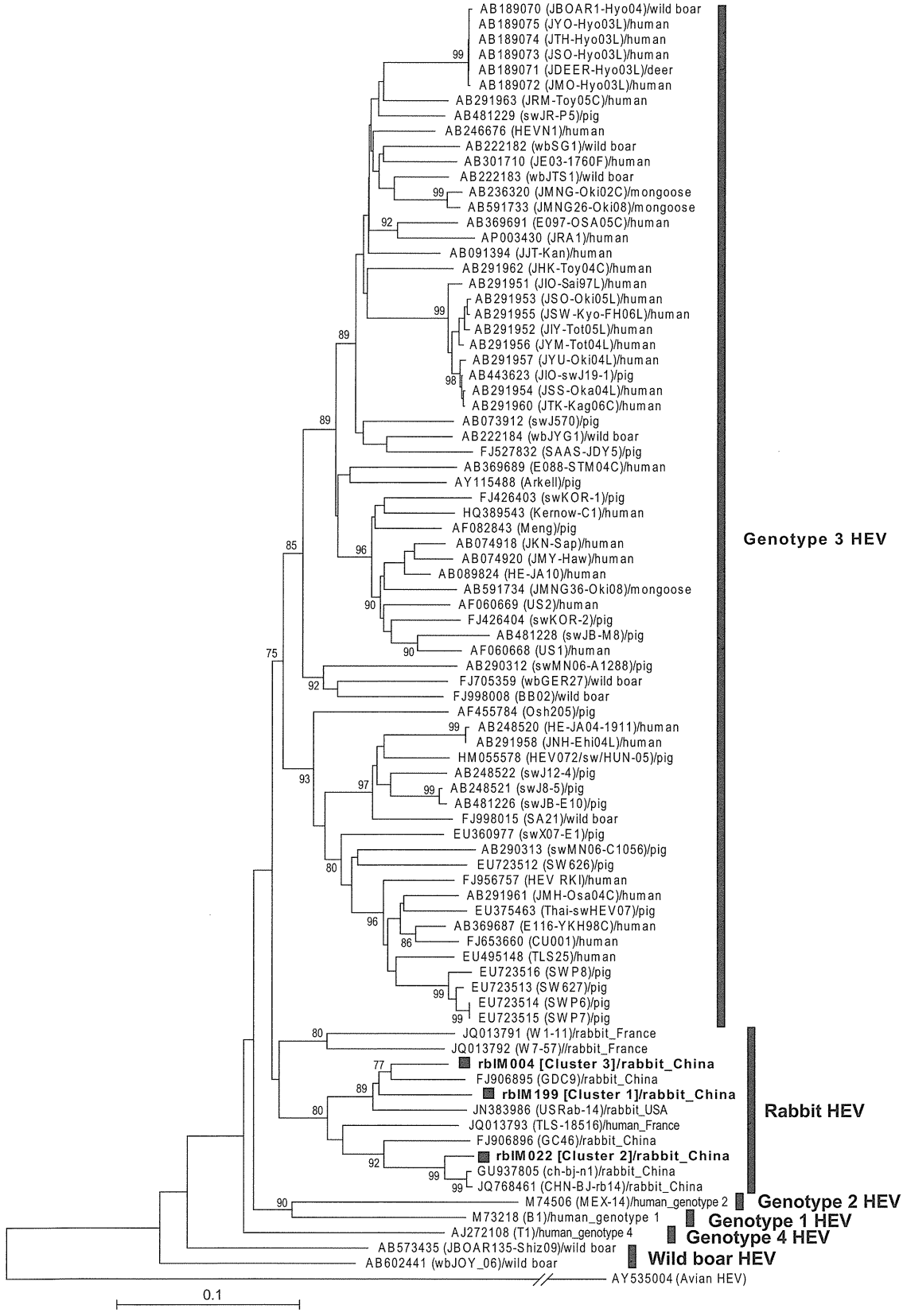

Supplementary Figure 1

The neighbor-joining tree of the 765-nt ORF2 sequence alignment containing 10 rabbit HEV strains, 73 HEV strains from humans, pigs, wild boars, deer, or mongoose, and an outgroup strain. In addition to the three Inner Mongolian rabbit HEV strains whose entire genomic sequences were determined in the present study, and which are indicated in boldface type and highlighted with closed boxes for visual clarity, all seven previously reported rabbit HEV strains whose overlapping 765-nt sequences were known and all HEV strains of genotype 3, as well as one representative HEV strain each of genotypes 1, 2, and 4, one HEV strain (JQ013793) recovered from a patient with hepatitis E in France that segregates into a cluster of rabbit HEV, and two wild boar HEV strains that may be classifiable into novel genotypes, were included for comparison. The reported strains are indicated with the accession no. followed by the name of the isolate in parenthesis and the name of the infected host from which it was isolated. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings. The scale bar indicates the nucleotide substitutions per site.

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Molecular epidemiology and genetic history of European-type genotype 3 hepatitis E virus indigenized in the central region of Japan ☆

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ABSTRACT

In Mie prefecture in Japan, 12 cases of sporadic hepatitis E occurred from 2004 to 2011. Mie prefecture is located in the central region of Japan, far from the most prevalent regions of hepatitis E virus (HEV) infection in Japan, the north and northeastern part. These 12 cases did not have any common risk factors of HEV infection. We analyzed the molecular epidemiology of the cases in Mie prefecture. We obtained the nucleotide sequences of the HEV strains and analyzed them with the sequences of other HEV strains by phylogenetic and coalescent analyses. Japan-indigenous genotype 3 HEV strains were divided into two major subtypes, namely, 3a and 3b; one minor subtype, 3e; and a few other unassigned lineages. The Japan-indigenous subtype 3e strains were closely related to European subtype 3e HEV strains and were comparatively rare in Japan; however, eight strains of the 12 cases we examined belonged to subtype 3e, indicating a close phylogenetic relationship, despite the lack of common risk factors. Coalescent analyses indicated that the Mie 3e strains seemed to have intruded into Mie prefecture about 10 years ago. Sporadic acute hepatitis E cases caused by the 3e strains occurred consistently from 2004 to 2011 in Mie prefecture. This is the first report of unexpected persistent occurrence of hepatitis by the European-type genotype 3 HEV, subtype 3e, in a country outside of Europe. Phylogenetic and coalescent analyses traced the history of the indigenization of the Mie 3e strains from Europe. Because hepatitis E cases caused by 3e strains are relatively rare in Japan, molecular evolutionary analyses of HEV infection in Mie prefecture is important for preventing a future hepatitis endemic or epidemic by 3e strains in Japan.

1. Introduction

Hepatitis E virus (HEV) is a positive-sense, single-stranded RNA virus consisting of approximately 7200 nt. It is a member of the genus *Hepevirus* in the *Hepeviridae* family (Emerson et al., 2005). The genome contains three open reading frames (ORFs), namely, ORF1, ORF2, and ORF3 (Chandra et al., 2008; Emerson et al., 2010; Yamada et al., 2009). HEV is the causative agent of hepatitis E. Outbreaks of hepatitis E generally occur in developing countries,

Abbreviations: BSP, Bayesian skyline plot; MCMC, Markov chain Monte Carlo; BF, Bayes factor; TMRCA, time of the most recent common ancestor.

☆ The GenBank/EMBL/DDBJ accession numbers of new genotype 3 HEV sequences determined in this study are AB607887, AB607889, AB607891, AB607892, AB607893, AB670119, AB670120, AB670123, and AB670124.

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where water sources are sometimes contaminated by feces containing HEV (Bradly et al., 1993; Okamoto, 2007); however, sporadic cases of hepatitis E have been reported in industrialized countries as well. Evidence from Japan and Europe have shown that HEV can be zoonotically transmitted to humans through the consumption of meat or offal of swine, wild boar, and deer infected with HEV (Bouwknegt et al., 2007; Reuter et al., 2009; Tamada et al., 2004; Tei et al., 2004; Yazaki et al., 2003).

Global HEV strains have been segregated into four genotypes, numbered 1–4 (Schlauder and Mushahwar, 2001). Lu et al. proposed further subtyping of each genotype through phylogenetic analyses (Lu et al., 2006). Recently, candidates for new genotypes were reported from rats (Johne et al., 2010) and rabbits in China (Zhao et al., 2009), and wild boars in Japan (Takahashi et al., 2010a, 2011). Genotypes 1 and 2 infect only humans and are associated with hepatitis E outbreaks in developing countries (Bradly et al., 1993; Okamoto, 2007), whereas genotype 3 strains have been isolated all over the world (Kaba et al., 2010; Lu et al., 2006), and genotype 4 cases have been reported in Asia, mainly in Japan and China (Lu et al., 2006). In a report involving 254 cases of Japanese hepatitis E cases, most of the cases were sporadic cases by genotypes 3 and 4 (Abe et al., 2006), and water-born outbreaks by genotype 1 or 2 have not been reported in Japan. The etiology of hepatitis E caused by genotypes 3 and 4 can be complex. In the above-mentioned study in Japan (Abe et al., 2006), some cases were caused by the ingestion of food products from pigs, boars, and deer infected with genotype 3 or 4 HEV strains. However, ~60% of the cases had no clear risk factors (i.e., travel to endemic area, transfusion, eating uncooked or undercooked mammalian liver or colon) for HEV infection, and the etiology remains to be determined. In addition, the contributions that the newly discovered genotypes are making to human disease are also unknown.

A nationwide survey revealed that genotype 3 is the most prevalent HEV genotype infecting humans (Sakata et al., 2008; Takahashi et al., 2010b) and swine (Takahashi et al., 2003) in Japan. Mizuo et al. first reported that multiple lineages of HEV genotype 3 are co-circulating in Japan, and these contribute to the development of sporadic acute hepatitis, with the prevalence differing according to age, sex, and geographic region (Mizuo et al., 2002). In our previous study, we reported that Japan-indigenous genotype 3 HEV was divided into at least three lineages, and that the European strains also originated from several lineages (Nakano et al., 2012).

Since November 2003, doctors in Japan are required to report all cases of hepatitis E to the local health bureau. The details of all reported cases can be found at the National Infectious Disease Surveillance Center in Japan (http://idsc.nih.go.jp/index-j.html). Around 41–71 cases have been reported in Japan every year from 2004 to 2011. Japan consists of four major islands: Hokkaido, Honshu (the main island), Shikoku, and Kyushu, situated from northeast to southwest (Fig. 1a). Mie prefecture is located in central region of the Honshu (Fig. 1a), far from the most prevalent regions of hepatitis E infection in Japan, which occur in the northeastern part of Honshu and in Hokkaido (Abe et al., 2006; Inoue et al., 2009b; Sainokami et al., 2004). In Mie prefecture, 0–4 cases of hepatitis E were reported annually, and a total of 16 cases have been reported from 2003 to 2011. We obtained serum samples from

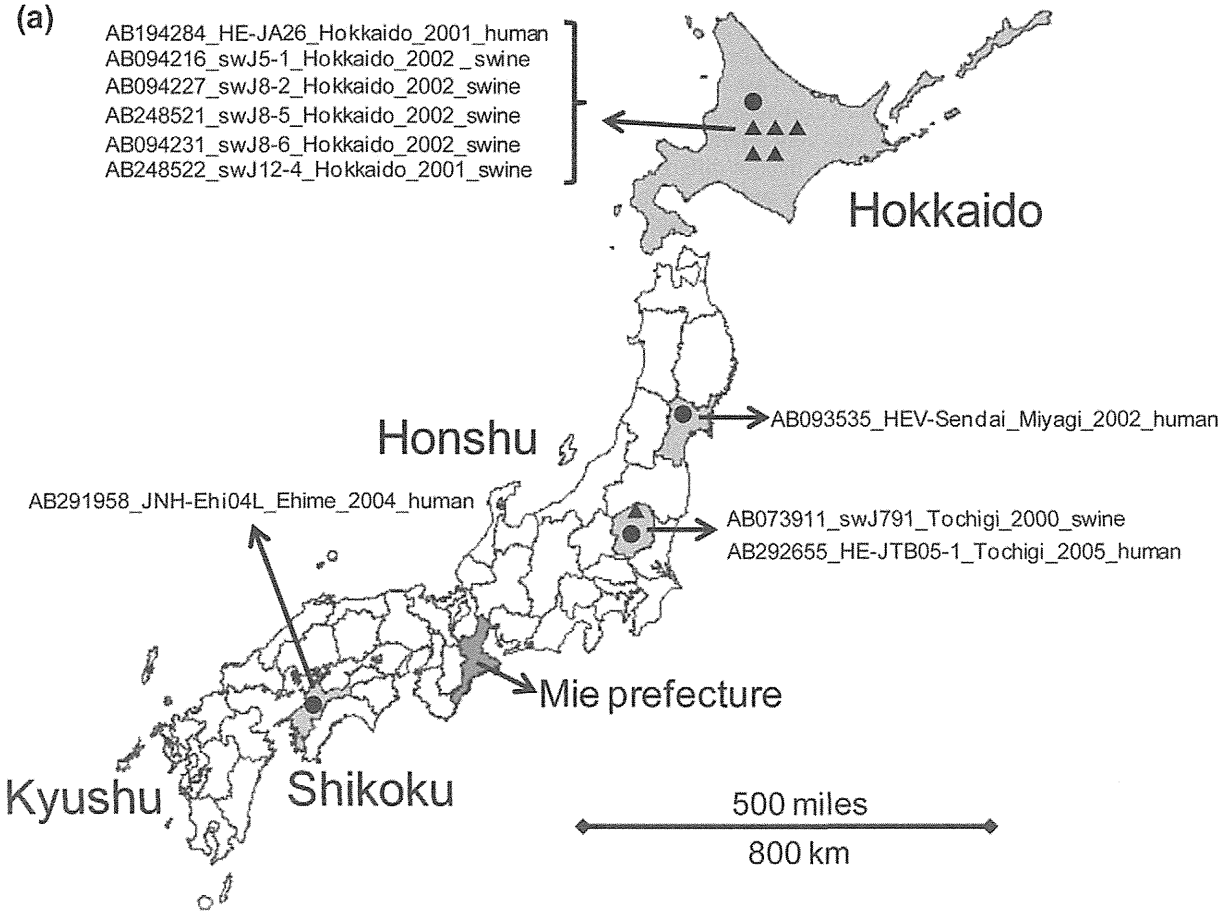

Fig. 1a. Map showing the four major islands of Japan, namely, Hokkaido, Honshu, Shikoku, and Kyushu, and the 47 prefectures. Gray indicates prefectures where the European-type genotype 3 HEV 3e strains were isolated. Black circles and triangles indicate strains isolated from human and swine, respectively. Accession numbers, strains' names, prefectures, years of isolation, and hosts are indicated at the tip of each arrow. Mie prefecture, which is investigated in this study, is indicated, with the details in Fig. 1b.

12 of these cases and analyzed the nucleotide sequences of the virus in 10 cases by phylogenetic trees.

Recently, epidemic histories of RNA viruses have been analyzed by coalescent-based methods using viral sequences. The method has been further improved by the addition of relaxed clock evolutionary models that incorporate variation in evolutionary rates, as well as with more advanced Bayesian inference methods that explicitly incorporate phylogenetic uncertainty (Drummond et al., 2006). Using these techniques, the epidemic histories of rabies in Brazil (Kobayashi et al., 2011), hepatitis A in France (Moratorio et al., 2007), hepatitis C in West Africa (Markov et al., 2009) and China (Nakano et al., 2006a), worldwide HEV (Purdy and Khudyakov, 2010), and HEV subtype 3a or 3b (Nakano et al., 2012) have been elucidated. We applied this technique to the viral sequences of our acute hepatitis E cases occurring in Mie prefecture and clarified the genetic history of the causative strains of HEV.

2. Material and methods

2.1. Sampling, isolation, and sequencing of HEV from sporadic acute hepatitis cases in Mie prefecture, Japan

We encountered 12 acute hepatitis E cases at five different hospitals (A to E) in Mie prefecture, Japan, from 2004 through 2011 (Table 1 and Fig. 1b). The patients presented with clinical features of acute hepatitis or elevated levels of liver transaminase, and were diagnosed with acute hepatitis E based on the detection of HEV RNA and/or HEV IgG and IgM antibodies (Inoue et al., 2006b; Mizuo et al., 2002; Nakano et al., 2006b). These 12 cases were deemed sporadic by detailed interviews that did not find any common risk factors of HEV infection, such as consuming uncooked or undercooked meat of wild mammals (boar or deer). Case 6 ate the liver of an unknown animal, and case 8 ate pork liver several weeks before the illness, but the onset of the cases was very different, the patients lived in different areas, and they ate the liver at different restaurants. Serum samples were obtained from the patients upon admission and stored at −20 °C or below until testing. The study was approved by the institutional review committee of Fujita Health University Nanakuri Sanatorium.

Case 1 was diagnosed by the presence of HEV IgM antibodies, but the serum sample was not stored for further analysis. We previously amplified and sequenced the full genome sequence of HEV of case 2 (Inoue et al., 2006b). We had also tested the serum of cases 3, 4, 5, and 6 and successfully amplified and sequenced the partial ORF1 region of HEV RNA (324 nt: nucleotide number 127–450 of the reference sequence **M73218) for cases 3, 5, and 6 (Nakano et al., 2006b). Serum from case 6 still remained and was analyzed again in the present study with the serum from cases 7 to 12. In cases 6–12, HEV RNA was amplified, and subsequently sequenced for both the ORF2 region (412 nt: nucleotide number 5944–6355) and the ORF1 region (412 nt: nucleotide number 39–450) by the method described previously (Mizuo et al., 2002). We had reported the ORF2 region sequences of cases 9, 10, and 11 in a case report (Okano et al., 2011). New nucleotide sequences determined in this study are the 412 nt-ORF2 region of cases 6, 7, 8, and 12 and the 412 nt-ORF1 region of cases 6–12. As a result, the nucleotide sequences of the partial ORF2 region and partial ORF1 region were available for sequence analyses in 8 and 10 cases, respectively (Table 1). These nucleotide sequences were analyzed with the HEV sequences retrieved from the DNA databases for the subsequent phylogenetic and coalescent analyses.

2.2. ORF2 and ORF1 data sets for phylogenetic tree analyses

All available nucleotide sequences of the 301 nt of ORF2 (nucleotide number 5994–6294: M73218) and the 287 nt of ORF1 (nucleotide number 104–390) were downloaded from the Hepatitis Virus Database using map viewer (http://s2as02.genes.nig.ac.jp/index.html). These two regions had been thoroughly sequenced and frequently used for genotyping and subtyping since Mizuo et al. compared Japanese HEV strains with global HEV strains (Mizuo et al., 2002). Lu et al. also used these regions when genotyping and subtyping global HEV strains by phylogenetic analyses (Lu et al., 2006). Database sequences were divided into each genotype by phylogenetic analyses, in which the database sequences were compared with the reference sequences of each genotype: genotype 1, M73218; genotype 2, M74506; genotype 3, AB073912; genotype 4, AB074915; genotype 5, AB573435 and AB576193 (Takahashi et al., 2010a); a newly reported novel genotype, AB602441 (Takahashi et al., 2011); and Chinese rabbit HEV, FJ906895 (Zhao et al., 2009).

Using SeaView version 4 (Gouy et al., 2010), we estimated the large trees by using neighbor-joining (under the Kimura 2-parameter substitution model). Only genotype 3 sequences isolated from Japanese patients, swine, and wild mammals, and the above-mentioned reference sequences were retained. In addition, only one sequence from each infected individual was retained. When several closely related sequences were isolated from a single outbreak of hepatitis E with the same origin, only one sequence was retained as the representative. Identical or very similar sequences whose epidemiological linkage was not certified were retained. Very similar sequences, with similarity values of >99.0%, isolated from a herd of swine were also excluded, except one sequence as the representative. Experimental clones that were not directly

Table 1
Acute hepatitis E cases occurring in Mie prefecture from 2004 to 2011.

Case No.	Year	Age	Gender	Hospital	Risk factor	Strain name	Analyzed region(s)	Type
Case 1	2004	66	M	A	None	na	na	?
Case 2	2004	51	M	B	None	HE-JA04-1911 [a,c]	ORF2 and ORF1	3e
Case 3	2005	55	M	C	None	248Ku55M [b]	ORF1	3e
Case 4	2005	66	F	C	None	na [b]	na	?
Case 5	2005	59	M	D	None	250Yo59M [b]	ORF1	3e
Case 6	2005	54	M	E	Zoonosis?	HE-JA05-0753 [b]	ORF2 and ORF1	3b
Case 7	2007	58	M	A	Travel	HE-JA07-0229	ORF2 and ORF1	4
Case 8	2007	55	M	D	Zoonosis?	HE-JA10-0841	ORF2 and ORF1	3e
Case 9	2008	46	M	A	None	HE-JA09-0135 [c]	ORF2 and ORF1	3e
Case 10	2009	61	M	A	None	HE-JA09-0195 [c]	ORF2 and ORF1	3e
Case 11	2010	67	M	A	None	HE-JA10-1071 [c]	ORF2 and ORF1	3e
Case 12	2011	66	M	A	None	HE-JA11-0494	ORF2 and ORF1	3e

"na" indicates "not applicable".
[a] We previously sequenced the full genome of the HEV of the case (Inoue et al., 2006b).
[b] We previously reported these cases as a case report (Nakano et al., 2006b).
[c] We previously sequenced these cases as a case report (Okano et al., 2011).

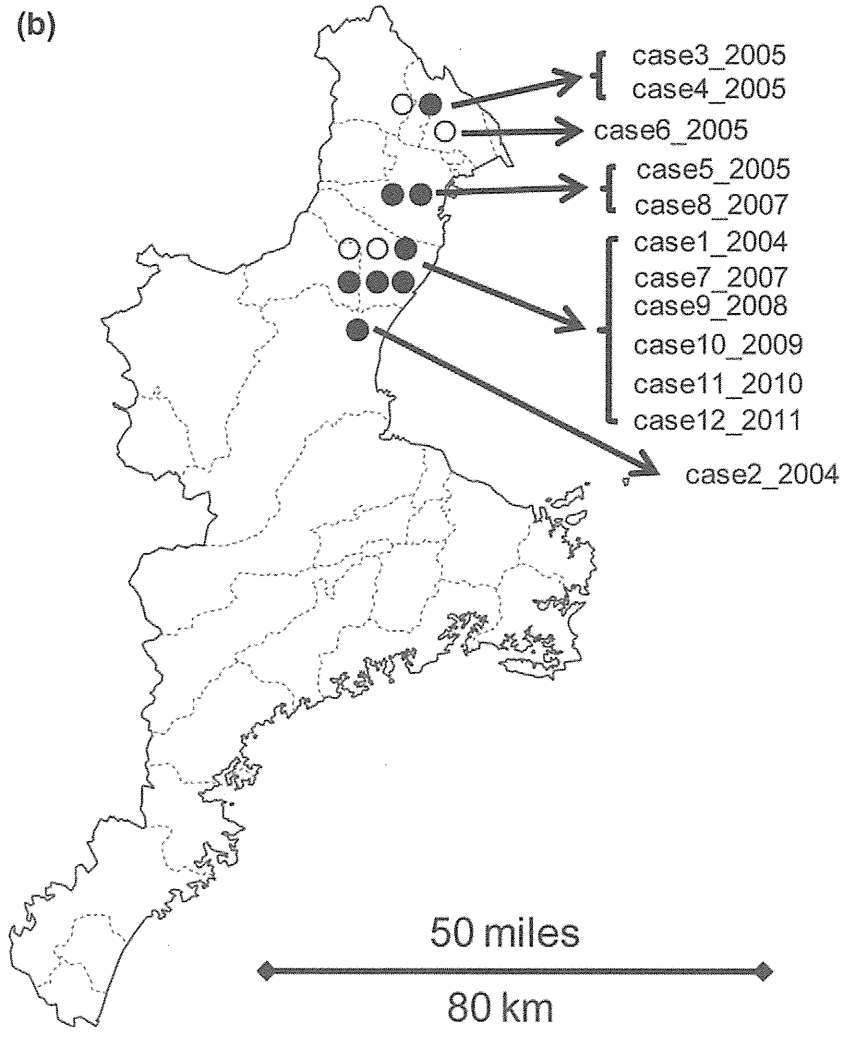

Fig. 1b. Map of Mie prefecture and the location of the occurrences of acute hepatitis E. Black circles indicate cases by 3e strains, and white circles, cases by other genotypes or subtypes. These 12 cases occurred in five hospitals (see Table 1). Case number and year of isolation are indicated at the tip of each arrow.

isolated from infected individuals were excluded. For the subtyping in the phylogenetic tree analyses, tentatively subtyped global strains described by Lu et al. were added to the ORF2 and ORF1 data sets as representatives of each subtype of genotype 3 (Lu et al., 2006). As described in detail in the Results section, most of the strains in the present study were closely related to the 3e strains tentatively assigned by Lu et al.; however, that study examined only a limited number of 3e strains, which did not provide a strong basis for comparison with the 3e strains that we studied. For more detailed phylogenetic analyses to address the relationship between our 3e strains and global 3e-related strains, we collected more 3e-related strains by using a blast search at DDBJ (http://www.ddbj.nig.ac.jp/Welcome-j.html) applying the ORF2–301-nt fragment and the ORF1–287-nt fragment of one of our 3e strains, HE-JA04-1911 (AB248520), as a query sequence. As a result, 27 sequences and 3 sequences, which have homology of >90% with the query sequence, could be added for the ORF2 and ORF1 data sets, respectively. Database sequences were then collated and aligned with our sequences, and were subsequently adjusted by hand. The resulting ORF2 alignment contained 227 Japanese genotype 3 sequences including 7 sequences of the present study, 70 foreign genotype 3 sequences, and 6 outgroup sequences. The resulting ORF1 alignment contained 104 Japanese genotype 3 sequences including our 9 sequences of the present study, 38 foreign genotype 3 sequences, and 6 outgroup sequences.

2.3. Phylogenetic tree analyses

We constructed neighbor-joining trees and maximum likelihood (ML) phylogenies to evaluate the phylogenetic clustering of the ORF2 and ORF1 data sets by using SeaView version 4. ML phylogenies were heuristically searched using the subtree pruning and regrafting (SPR) and nearest neighbor interchange (NNI) perturbation algorithms. The statistical robustness levels of phylogenetic groupings were subsequently assessed using bootstrap analyses (1000 replicates for neighbor-joining trees and 500 replicates for ML phylogenies). Phylogeographic structure was then identified using FigTree (available from http://tree.bio.ed.ac.uk), and ancestral lineage states of clades and lineages of Japanese strains were colored by using a parsimony approach (Slatkin and Maddison, 1989). The colors were used according to the subtyping tentatively determined by Lu et al. (2006).

2.4. Coalescent analyses of Japan-indigenous genotype 3 HEV strains

Including seven genotype 3 sequences of the 412 nt of the ORF2 region (nucleotide number 5944–6355) of the present study, 185 Japan-indigenous genotype 3 ORF2 sequences were available as sequences with known sampling dates. The sampling dates of the 412 nt of the ORF2 data set ranged from 1979/10/9 (AB175483) to 2011/3/24 (AB670124). From the dated sequence data sets, we

inferred the genetic history of Japan-indigenous genotype 3 sequences by using the framework of coalescent analysis implemented in the program package BEAST (Drummond and Rambaut, 2007). Markov chain Monte Carlo (MCMC) sampling was performed for at least 2×10^8 generations, sampling a tree every 10,000 generations. We used the general time-reversible model of nucleotide substitution, with rate heterogeneity among sites modeled using a discrete gamma distribution with four rate categories. To select the best-fitting molecular clock and demographic model, we calculated the marginal likelihoods of the data conditional on all evolutionary and demographic model parameters. We analyzed all possible combinations of the relaxed (Drummond et al., 2006) and strict molecular clock models and of the Bayesian skyline (Pybus et al., 2003), constant, exponential, logistic, and expansion growth coalescent models. As a result, we estimated Bayes factors (BFs) for each pair of models to choose the most statistically appropriate model (Suchard et al., 2001). The program Tracer (http://tree.bio.ed.ac.uk) was used to estimate BFs, to check for convergence, and to determine whether appropriate mixing of the posterior target distribution had been achieved (effective sample size >200).

A Bayesian estimate of phylogeny was obtained from the posterior distribution of trees arising from the best-fitting BEAST analysis (see above). First, the program TreeAnnotator (Drummond and Rambaut, 2007) was used to construct a phylogeny that best summarizes the set of credible trees, called the maximum clade support phylogeny. Because a relaxed clock was used in the Bayesian MCMC analysis, the branch lengths and node heights of the maximum clade support phylogeny are in units of years (Drummond et al., 2006). Phylogeographic structure was then identified using FigTree.

2.5. Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers of new genotype 3 HEV sequences determined in this study are AB607887, AB607889, AB607891, AB607892, AB607893, AB670119, AB670120, AB670123, and AB670124.

3. Results

3.1. Comparatively minor Japan-indigenous 3e strains are nested under European strains in phylogenetic trees

In the neighbor-joining tree of the 301-nt sequence within the ORF2 region, the subtypes of clusters were guided by following the subtyping set forth by Lu et al. (2006) (Fig. 2). According to the increasing numbers of the sequences, the structure of subtyping by Lu et al. became ambiguous. No significant bootstrap support was obtained for each subtype. Similar results were obtained in the ML tree (data not shown). In spite of the ambiguity of the subtyping in the large phylogeny of the short sequences, the structure was generally consistent with that of the small phylogeny based on corresponding full genome sequences (data not shown). Then, 297 genotype 3 strains were tentatively divided into subtypes 3a, 3b, 3c, 3d, 3e, 3f, 3g, and undetermined. A 3j strain (AY115488) isolated from Canada was included in the 3b cluster in this tree, possibly because of the weak phylogenetic signal of the short region (Fig. 2b).

Of the 227 Japanese strains, 138 strains belonged to the 3b cluster (Fig. 2b), 58 strains belonged to the 3a cluster (Fig. 2c), 21 strains belonged to the 3e cluster (Fig. 2a), and two strains belonged to the 3f cluster (Fig. 2a). The two Japanese 3f strains were strongly suggested to be imported HEV strains after traveling to endemic area (Miyaji et al., 2005) (published only online). We could not determine the subtype of eight Japanese strains due to the absence of the appropriate guiding sequences in the subtyping done by Lu et al. (Fig. 2a). The 21 Japanese 3e strains were further separated into three lineages by European 3e strains (Fig. 2a).

In the neighbor-joining tree of the 287-nt sequence in the ORF1 region, subtyping by Lu et al. also served as a provisional standard (Fig. 3). In this tree, as well as in the ML tree, the structure of the subtyping also became ambiguous, and no significant bootstrap support was obtained for each subtype in either tree (data not shown). However, the structure was generally consistent with that of the above-mentioned ORF2 phylogeny. In the tree of ORF1, 142 genotype 3 strains were divided into subtypes 3a, 3b, 3c, 3e, 3f, 3g, 3h, 3i, 3j, and undetermined (Fig. 3). Out of 104 Japanese strains, 65 strains belonged to the 3b cluster, 23 strains belonged to the 3a cluster, 12 strains belonged to the 3e cluster, and two strains belonged to the 3f cluster. The two Japanese 3f strains were the same strains as those in the ORF2 tree, that is, they were imported cases. We could not determine the subtype of two Japanese strains because of the absence of the appropriate guiding sequences in the subtyping done by Lu et al. (Fig. 3).

From the results of the ORF2 and ORF1 region trees, Japan-indigenous genotype 3 strains were divided into two major clusters, 3a and 3b, and one minor cluster, 3e, with very few unassigned strains. The Japan-indigenous 3e strains were comparatively minor in Japan and were nested under European 3e strains in the trees.

3.2. Persistent occurrence of sporadic acute hepatitis E by European-type genotype 3 HEV in Mie prefecture

Of the 12 HEV cases in Mie prefecture (Table 1), neither ORF2 nor ORF1 nucleotide sequences were obtained for two cases (cases 1 and 4). Nucleotide sequences of the partial ORF2 and partial ORF1 region were available for eight cases and 10 cases, respectively. Case 7 was typed as genotype 4 in both the ORF2 and ORF1 trees (data not shown). The case had a clear risk factor: traveling to China within 6 weeks of the onset of the illness. In the ORF2 region tree, seven strains were typed as genotype 3 (Fig. 2). Case 6 was typed as subtype 3b, which is the most common subtype in Japan (Fig. 2b). The other six genotype 3 Mie strains were included in one of the 3e lineages and were closely related to one another (Fig. 2a). Simple homology of the 412-nt ORF2 region among the six ORF2 3e strains ranged from 98.5% to 99.8%. In the ORF1 region tree, nine strains were typed as genotype 3 (Fig. 3). Case 6 was typed as subtype 3b as in the ORF2 tree. The remaining eight genotype 3 Mie strains were included in the 3e cluster and indicated close relationship with one another. Simple homology of the 412-nt (or 324-nt) ORF1 region among the eight ORF1 3e strains ranged from 98.1% to 99.8%. Another strain from the Ehime prefecture in Shikoku also clustered together with the Mie 3e strains in the trees.

These data show that, although 3e strains are relatively minor compared to other genotype 3 strains in Japan, eight strains of the 12 cases that we analyzed from Mie prefecture belonged to subtype 3e (Table 1). Despite having no common risk factor (Table 1), phylogenetic trees and sequence homology indicate a close relationship between these strains. Judging from the structure of the two trees, these Mie 3e strains seem to originate from one of the European-type genotype 3 lineages, 3e. Our analyses have shown that sporadic acute hepatitis E cases caused by the comparatively minor Japan-indigenous 3e lineage occurred not frequently but persistently in the Mie prefecture from 2004 to 2011.

3.3. Estimation of time of the most recent common ancestors (TMRCAs) of Japan-indigenous 3e and Mie 3e strains by using the ORF2 region data set

Evolutionary analysis of the dated ORF2 region data set was performed in BEAST under a range of molecular clock and coalescent

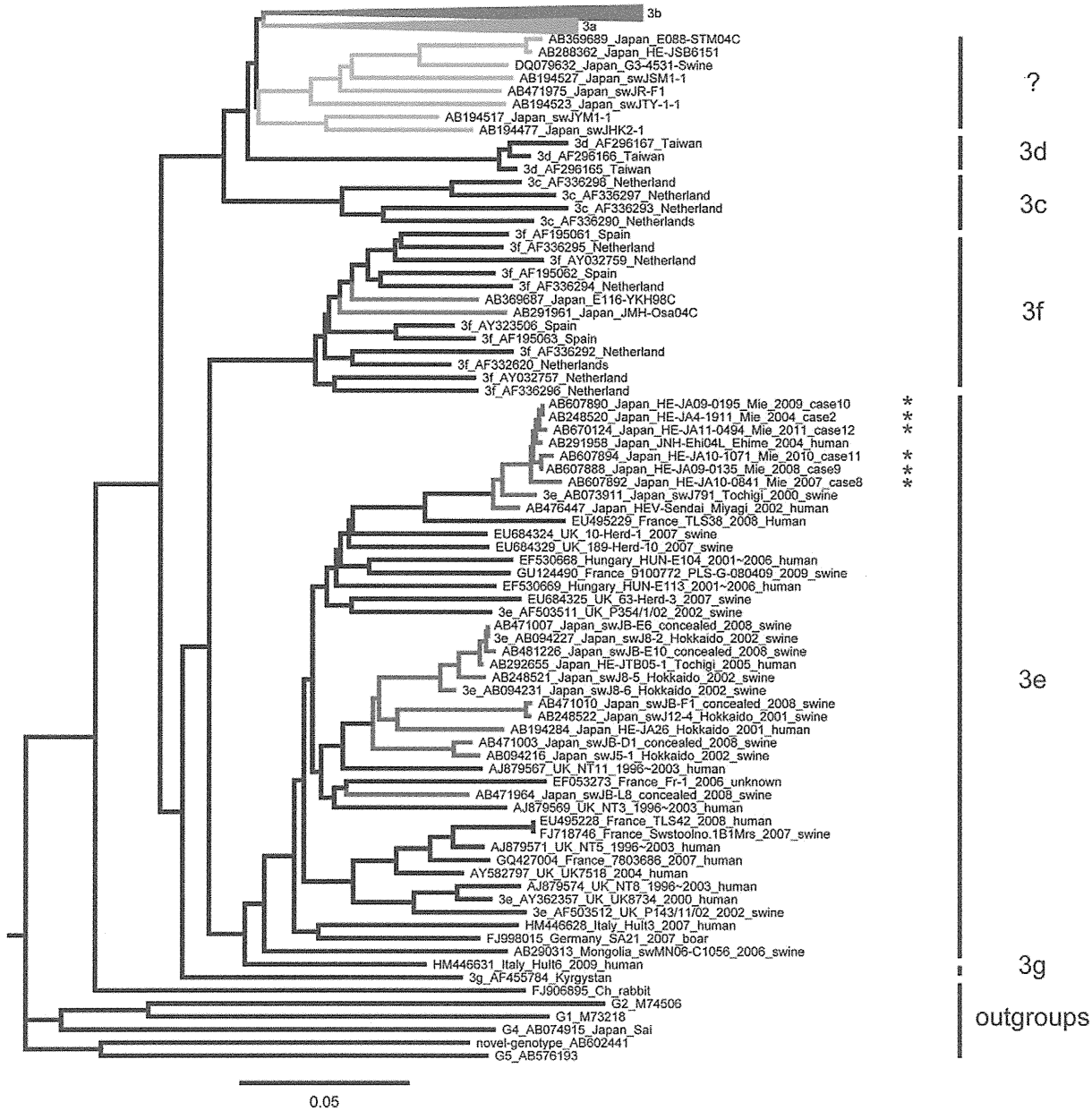

Fig. 2. The neighbor-joining tree of 301 nt of ORF2 alignment containing 227 genotype 3 strains isolated in Japan and the outgroup strains. Since it was difficult to indicate all strains in one figure due to the large number of sequences, clusters of subtypes 3b and 3a clusters are collapsed in (a) and are shown in (b) and (c), respectively. Clusters of other subtypes and outgroup strains are shown in (a). Sequences in the literature of Lu et al. are included as guides for subtyping (Lu et al., 2006). Subtypes of those sequences are shown at the top of the strain names. The ancestral lineage states of clades are indicated using a parsimony approach. The lineages of Japanese strains are colored: 3a, green; 3b, red; 3e, blue; 3f, brown; undetermined lineages, cyan. The lineages from other countries remain black. The origin of each strain is also indicated by the strain names. The strains isolated in Mie prefecture are indicated by * following the strain name. The scale bars indicates nucleotide substitutions site^{-1}.

model combinations. The combination of three clock models and BSP gave similar median estimates of evolutionary rate and TMRCAs of Japan-indigenous 3e as well as Mie and Ehime 3e clusters (Table 2). The strict clock model and uncorrelated lognormal clock gave a narrow range for the evolutionary rate: 1.34×10^{-3} substitutions site^{-1} year^{-1} (95% credible interval (CI), 1.02×10^{-3} to 1.69×10^{-3}) and 1.34×10^{-3} (95% CI, 1.02×10^{-3} to 1.69×10^{-3}), respectively. The TMRCA using the strict clock model for Japan-indigenous 3e strains was 1963 (95% CI, 1944–1974), and for the Mie and Ehime 3e cluster was 2003 (95% CI, 2001–2004). The TMRCA using the uncorrelated lognormal clock model for Japan-indigenous 3e was 1964 (95% CI, 1948–1974), and for the Mie and Ehime 3e cluster was 2003 (95% CI, 2001–2004). However, the uncorrelated exponential clock model gave the best BF, and also a wider range of evolutionary rates and TMRCAs. The evolutionary rate was 1.49×10^{-3} substitutions site^{-1} year^{-1} (95% CI, 1.03×10^{-3} to 2.02×10^{-3}). The TMRCA for Japan-indigenous 3e strains was 1966 (95% CI, 1946–1982), and for Mie and Ehime 3e cluster was 2002 (95% CI, 1999–2004).

Fig. 4 shows the maximum clade support phylogeny for the dated ORF2 sequence set, reconstructed from the phylogenies sampled under the best supported model: the uncorrelated exponential clock model and BSP. Japan-indigenous 3e cluster strains and Mie and Ehime 3e cluster strains clustered together with high posterior probabilities. The Japan-indigenous 3e strains branched from the other subtypes at the oldest age with no more branching until after 1960. Mie and Ehime 3e strains started to branch after the year 2000, corresponding to the values from the TMRCA.

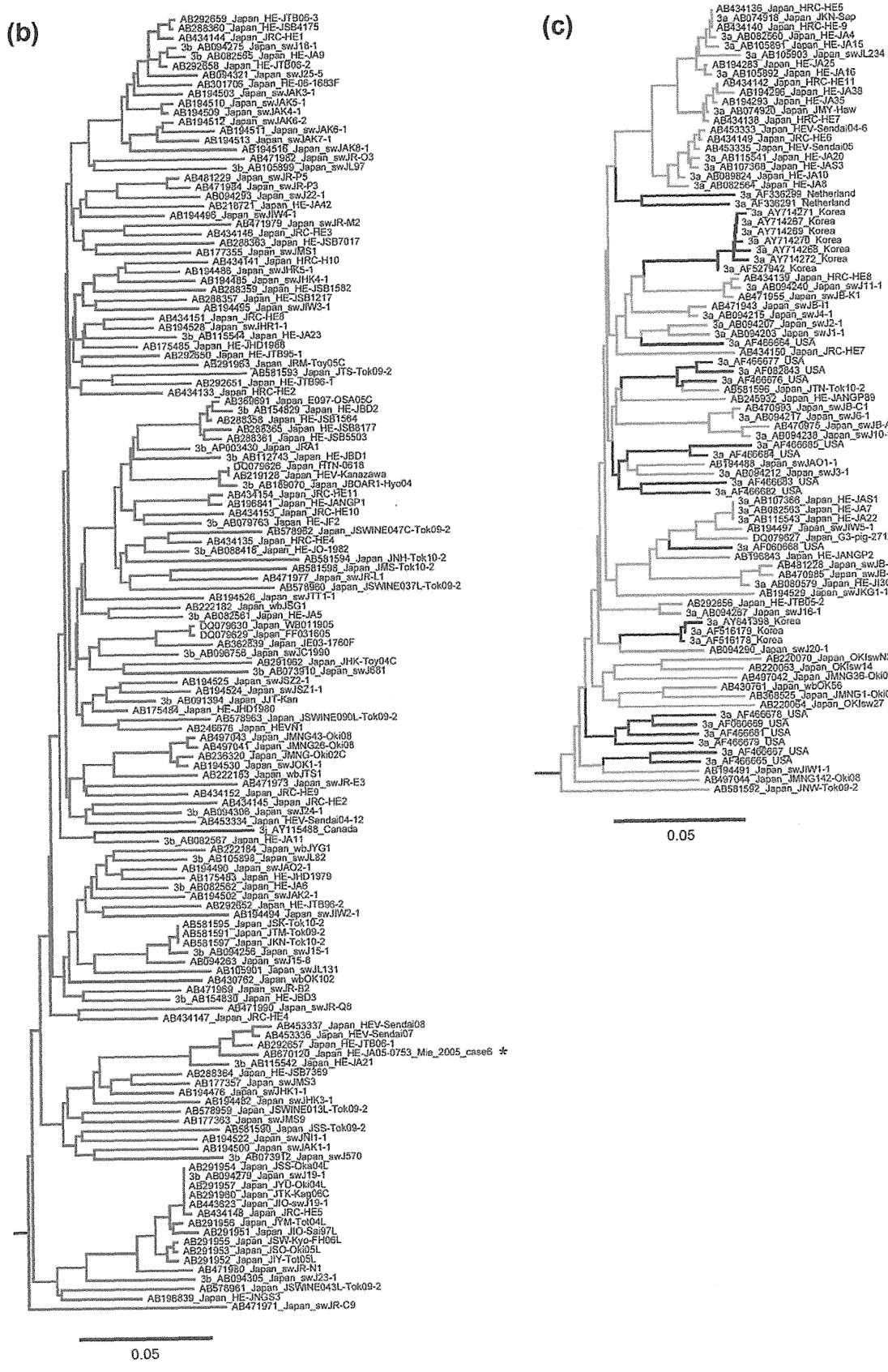

Fig. 2 (*continued*)