

was obtained from a Japanese patient who contracted a domestic HAV infection in 2010 as well as HAJNS-BorSap99 (AB618531) that was obtained from a Japanese patient infected in Kota Kinabalu of Borneo Island, Malaysia, in 1999,²³ supported by a bootstrap value of 100% (Fig. 1). Thereafter, due to the markedly high similarity (99.99% or 7476/7477) between the two isolates obtained in the present study (Table 2), HA12-0796 as a representative isolate was subjected to comparison with other reported HAV isolates.

Comparison of HA12-0796 with reported partial nucleotide sequences of subgenotype IA HAV isolates

Because the entire genomic sequence of subgenotype IA HAV in the Philippines, where our patients were presumed to have contracted the HAV infection while traveling, has not yet been reported, the HA12-0796 isolate obtained from patient 1 was compared with all 13 subgenotype IA HAV isolates whose partial nucleotide sequences of 198–237 nt were retrievable from the DDBJ/GenBank/EMBL databases (Table 2), all of which were recovered from water in urban rivers in metropolitan Manila, the Philippines, in 2009. HA12-0796 shared

the highest nucleotide sequence identity of 99.5% with a HAV isolate (AB567672) within an overlapping 228-nt sequence, and was 96.2–99.1% identical to the remaining 12 isolates, supporting the notion that patients 1 and 2 in the present study contracted the HAV infection in the Philippines.

To exclude the possibility that HA12-0796 and HA12-0938 originated from a country other than the Philippines, HA12-0796 was compared with reported HAV isolates of the same subgenotype in other Asian countries, whose entire or partial IA sequence of more than 200 nt within the coding region was known, including Japan, as well as China, Indonesia, Korea, Malaysia, Taiwan and Thailand, from which various fresh and perishable foods, such as seafood, are currently imported to Japan (Table 3). HA12-0796 shared only 93.4–96.9% identity within the 200–7431 nt sequence with the isolates from China, Indonesia, Korea, Taiwan and Thailand, and at the highest, had only 98.2% identity over the entire genome with the HAV isolate (HAJNS-BorSap99 [AB618531]) imported from Malaysia, which was recovered from a Japanese patient with acute hepatitis A who had traveled to Kota Kinabalu on Borneo Island, Malaysia, in 1999.²³ With regard to the HAV isolates identified in Japan, 37 of 409 IA isolates

Table 2 Comparison of the HA12-0796 isolate obtained from patient 1, whose entire genomic sequence was determined in this study, with the HA12-0938 isolate obtained from patient 2, who traveled to the Philippines with patient 1, and 13 HAV isolates recovered from river water in the Philippines

| Isolate name or accession number | Sampling location | Collection date | Nucleotide position (length) compared | Identity% | |
|--|--------------------------------------|-----------------|---------------------------------------|-------------|-------------|
| | | | | Nucleotides | Amino acids |
| HAV isolate from Patient 2 | | | | | |
| HA12-0938 | Japan | July 2012 | 1–7477 (7477)† | 99.9 | 100 |
| HAV isolates recovered from river water in the Philippines | | | | | |
| AB567666 | Luzon, Metro Manila | March 2009 | 2930–3166 (237) | 96.6 | 98.7 |
| AB567667 | Luzon, Metro Manila | April 2009 | 2930–3157 (228) | 96.9 | 98.6 |
| AB567668 | Luzon, Metro Manila | April 2009 | 2930–3127 (198) | 97.4 | 98.7 |
| AB567669 | Luzon, Metro Manila | April 2009 | 2930–3157 (228) | 96.4 | 98.6 |
| AB567670 | Luzon, Metro Manila, Las Pinao River | April 2009 | 2930–3160 (231) | 96.9 | 100 |
| AB567671 | Luzon, Metro Manila, Paranaque River | April 2009 | 2930–3166 (237) | 97.8 | 100 |
| AB567672 | Luzon, Metro Manila, San Juan River | April 2009 | 2930–3157 (228) | 99.5 | 100 |
| AB567673 | Luzon, Metro Manila | August 2009 | 2930–3166 (237) | 98.3 | 100 |
| AB567674 | Luzon, Metro Manila, Paranaque River | November 2009 | 2930–3166 (237) | 99.1 | 100 |
| AB567675 | Luzon, Metro Manila | December 2009 | 2930–3166 (237) | 98.3 | 100 |
| AB567676 | Luzon, Metro Manila | December 2009 | 2930–3166 (237) | 96.2 | 98.7 |
| AB567677 | Luzon, Metro Manila, Paranaque River | December 2009 | 2930–3166 (237) | 99.1 | 100 |
| AB567678 | Luzon, Metro Manila, Las Pinan River | April 2009 | 2930–3166 (237) | 96.6 | 98.7 |

†A poly(A) tract at the 3'-end was excluded.

HAV, hepatitis A virus; Metro, metropolitan.

Table 3 Comparison of the HA12-0796 isolate obtained from patient 1, whose entire genomic sequence was determined in this study, with Asian genotype IA HAV strains with a known genomic sequence of more than 200 nucleotides

| Country | Number of isolates compared | Length of nucleotide sequence compared | Nucleotide identity (%) |
|---------------|-----------------------------|--|-------------------------|
| Philippines | 12 | 228–237 | 96.2–99.5 |
| Japan | 409 | 232–7492 | 91.3–98.8 |
| IA-1 (Ishii)† | 11 | 232–475 | 93.3–96.1 |
| IA-2 (Ishii)† | 30 | 232–7455 | 97.5–98.8 |
| Others | 368 | 232–7492 | 91.3–98.5 |
| Malaysia | 1 | 7494 | 98.2 |
| Thailand | 10 | 200–358 | 94.5–96.9 |
| Korea | 42 | 390–2582 | 94.5–96.6 |
| China | 22 | 321–7431 | 93.7–96.1 |
| Indonesia | 10 | 481 | 94.5–96.0 |
| Taiwan | 83 | 481–855 | 93.4–96.0 |

†Two IA sublineages (IA-1 and IA-2) described by Ishii *et al.*¹⁵ HAV, hepatitis A virus.

(9.0%) exhibited nucleotide sequence identities of 98.1–98.8% with HA12-0796. However, there were no reported isolates in Japan that had high nucleotide sequence identities (>99.0%) with HA12-0796.

Figure 2 shows the neighbor-joining tree of the overlapping 220-nt sequence (nt 2938–3157) in the VP1/2A segment of 80 HAV isolates including those of Philippine and Japanese origins. Although no significant bootstrap support was obtained, possibly because of the weak phylogenetic signal of the short region, HA12-0796 and HA12-0938 were grouped into a cluster with three Philippine isolates (AB567672, AB567674 and AB567677) which shared the highest identities of 99.1–99.5% within the overlapping 228–237 nt sequence with HA12-0796 and HA12-0938 (Table 2). Although the bootstrap value was low at 56%, a larger cluster included these five isolates, as well as IA-2 sublineage HAV strains recovered from patients who were implicated in mini-epidemics of HAV in 2010 in Japan,¹⁵ and a Malaysian strain (AB618531) which was isolated from a Japanese patient with imported hepatitis A in 1999.²³

DISCUSSION

IN THE PRESENT study, we determined and analyzed the full-length genomic sequences of subgenotype IA HAV isolates that were recovered from two Japanese patients who developed acute hepatitis A 1 month after

travelling to the Province of Southern Leyte, the Philippines, to enjoy scuba diving. It may be possible that the patients contracted a domestic infection with Japan-indigenous HAV or HAV circulating in a country other than the Philippines through the consumption of imported foods, either before or after travelling to the Philippines, due to the fact that their trip was just a short-term tour lasting only 5 days. However, the two patients lived approximately 110 km apart in Japan and consumed the same foods and drinks only when they travelled to the Philippines. Nearly identical HAV isolates differing by only one nucleotide over the entire genome were recovered from the two patients, indicating that the two isolates were most likely derived from a common infectious source, such as food they ingested. In support of this notion, the patients independently reported to their physicians that they ate undercooked shellfish in the same restaurant while travelling in Southern Leyte. These patients developed acute hepatitis A 1 month after the consumption of shellfish, consistent with the known incubation period for hepatitis A ranging 10–50 days, with a mode of approximately 1 month.² Furthermore, the HAV isolates obtained from the two patients shared the highest nucleotide sequence identities of 99.1–99.5% with three HAV isolates (AB567672, AB567674 and AB567677) recovered from river water in the Philippines among all known HAV isolates deposited in the DDBJ/GenBank/EMBL databases as of January 2013. These results and observations indicate that the HA12-0796 and HA12-0938 isolates obtained from the patients in the present study represent a HAV strain circulating in the Philippines. Because no full-length genomic sequence data for HAV isolates of subgenotype IA in the Philippines have been determined thus far, the sequence data of the HA12-0796 and HA12-0938 isolates may be useful for molecular epidemiological studies on HAV infection as the prototype IA isolates circulating in this country.

The HAV endemicity level for a population is defined by the results of age-seroprevalence surveys. Recently, a systematic review on the global prevalence of HAV infection was published by the World Health Organization.²⁴ There is considerable variety in the seroprevalence rates in countries in South-East Asia, with some continuing to have high rates and others shifting from high to moderate and moderate to low endemicity, with a corresponding increase in the age of exposure from childhood to early adulthood. These changes have resulted from improvements in hygiene, sanitation and the quality of drinking water, reflecting improvements in living standards and socioeconomic progress.

In the Philippines in 1986, almost 100% of the rural populations had anti-HAV antibodies by the age of 15 years,²⁵ while only 48% of the residents at a similar age in the middle-income/middle-class communities in Manila were antibody positive, indicating regional variations in HAV seroprevalence in the Philippines.¹⁴ In 1992–1993, the overall HAV seroprevalence was 62% in metropolitan Manila, with age-related seroprevalence which increased progressively with age, and adults aged 40 years and over showed a seroprevalence of more than 90%.¹³ The age-related and overall seroprevalence in the urban areas in the 1990s appeared to reflect that of moderate endemicity, although there was little evidence identifying any change over the preceding decades. The main types of outbreaks in the Philippines are reported to be those in schools, attributable to the consumption of contaminated food or drinking water.²⁶ Although the change in HAV seroprevalence over time remains unclear, due to the absence of longitudinal studies assessing similar populations in the Philippines, the current urban Philippine populations may be typical of a country with moderate endemicity, while the seroprevalence in rural areas may be typical of high endemicity. Further studies via seroepidemiological and molecular virological approaches are clearly required to clarify the present status of HAV infection in this country in order to prevent HAV infection in not only residents, but also tourists from non-endemic countries, including Japan.

In Japan, the number of hepatitis A cases has been steadily decreasing since the 1990s due to improved sanitary conditions.²⁷ However, a low incidence of infection results in increased HAV susceptibility. Among 2430 Japanese individuals aged 0–92 years in 2003, the overall seroprevalence was 12.2%, with rare detection of anti-HAV antibodies in individuals aged 0–44 years and a gradual increase of seropositivity starting from the age of 45–49 years through age 65 years and above.²⁸ Notably, the prevalence of anti-HAV antibodies in individuals older than 50 years in 2003 was 50.3%, which is significantly lower than that of corresponding studies in 1994 (74.3%), 1984 (96.9%) and 1973 (96.9%).²⁸ Most of the infections that occurred in Japan were sporadic, with the rare occurrence of small-scale outbreaks.²⁹ Although the annual numbers of reported cases were low, at approximately 100–150 in the last several years, a total of 346 cases was reported in 2010 due to mini-epidemics in several regions of Japan, of which 61 cases were subjected to HAV genotyping, with IA (44 cases), IB (one case) and IIIA (16 cases) all being identified.¹⁵

Ishii *et al.*¹⁵ provisionally divided the isolated genotype IA strains into two sublineages, including one (IA-1) corresponding to strains endemic to Japan and another (IA-2) having sequences that were very similar to each other. Of note, 26 of the 31 IA-2 isolates were 100% identical,¹⁵ suggesting that some of the mini-epidemics occurred from a common infection source. Based on the phylogenetic analyses of the VP1/2A junction region from HAV isolates, it was suggested that the IA-2 sublineage may be connected with the Philippines.¹⁵ In support of this speculation, our IA isolates (HA12-0796 and HA12-0938), which are most likely representative Philippine isolates, shared 98.8% identity over the entire genome with one of the IA-2 isolates (HAJIH-Fukuo10 [AB618529]) which was recovered from a Japanese female who developed a domestic HAV infection in April 2010, together with her husband (HAJTH-Fukuo10; see Fig. 2).²³ In the phylogenetic tree constructed based on the entire genome, these three isolates (HA12-0796, HA12-0938 and HAJIH-Fukuo10) segregated into a cluster with a bootstrap value of 100%, and, interestingly, formed another cluster together with the HAJNS-BorSap99 isolate (AB618531) that was obtained from a patient infected on Kota Kinabalu of Borneo Island, Malaysia, located south to Mindanao Island, the Philippines,²³ which was supported by a bootstrap value of 100% (Fig. 1). During the mini-epidemics in 2010, the isolates belonging to the IA-2 sublineage were detected mainly from late March through May, and could not be detected after June.¹⁵ These results indicate that HAV isolates belonging to the IA-2 might have been imported from the Philippines. However, the limited number of patients studied, lack of direct evidence of infectivity in foods that might have been related to the disease of these patients and possibility of different routes of HAV infection should be taken into consideration. To draw a definitive conclusion, HAV isolates that are nearly identical to the IA-2 lineage isolates represented by HAJIH-Fukuo10 within the entire or longer partial genomic sequences should be identified in the Philippines in future studies.

In conclusion, the entire genomic sequences of the first Philippine-indigenous genotype IA HAV isolates recovered from two Japanese patients, who developed acute hepatitis A 1 month after traveling to the Philippines, were determined in the present study. These sequence data may be useful for molecular epidemiological studies on HAV infection in Asian countries, especially in the Philippines and Japan, in relation to the clarification of source(s) of HAV infection through the exchange of people and food products between

Japan and other countries. Because the seroepidemiological and molecular virological data on HAV infection in the Philippines are limited at present, the surveillance of anti-HAV antibody prevalence and analyses of HAV genomes implicated in acute hepatitis A are needed for implementing preventive measures and for controlling the spread of HAV in this country. Both patients in the present study had not received vaccination for HAV prior to traveling to the Philippines. Therefore, travelers to countries where HAV infection is moderately or highly endemic, like the Philippines, should receive pre-exposure prophylaxis with a hepatitis A vaccine or Ig before travel, unless they have previously been vaccinated or infected with HAV.

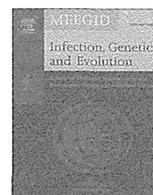
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Identification of European-type hepatitis E virus subtype 3e isolates in Japanese wild boars: Molecular tracing of HEV from swine to wild boars



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ABSTRACT

Nucleotide sequences of hepatitis E virus (HEV) isolates infecting wild boars in Mie prefecture, which is located in the central region of Japan and is far from the most prevalent regions of HEV infection in Japan, were determined and characterised. Among 144 serum samples of wild boars captured in Mie prefecture, 7 were positive for HEV-RNA. The nucleotide sequence of nearly the entire genome was determined for 4 of the 7 positive samples. Phylogenetic tree analyses indicated that 6 samples were subtype 3e and 1 was subtype 3a among the 7 isolates. We identified the indigenization of subtype 3e isolates in Japanese wild boars. Furthermore, 5 subtype 3e isolates were closely related and were located in the peripheral branch of subtype 3e isolates from European countries in the phylogenetic tree. The structure indicated that the ancestor of the 5 subtype 3e isolates originated in Europe. The phylogenetic structure and coalescent analyses suggested that the subtype 3e isolates entered Japan from Europe by importation of large-race pigs around 1966. The results also indicated that several lineages of subtype 3e expanded to a wide area of Japan around 1992 and 1 of the lineages was indigenized in wild boars in Mie prefecture between 1992 and 2009. The appearance of a wild boar cluster in the peripheral branch in the phylogenetic lineage may indicate the direction of gene flow of HEV subtype 3e from swine to wild boars. Clarification of the transmission direction or route should be helpful to prevent a future endemic or epidemic of HEV infection.

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1. Introduction

Hepatitis E virus (HEV) is a positive-sense, single-stranded RNA virus with a genome of approximately 7200 nt. It is a member of the genus *Hepevirus* in the *Hepeviridae* family (Anderson et al., 2011). The genome contains a short 5' untranslated region (UTR), 3 open reading frames (ORFs: ORF1, ORF2, and ORF3), and a short 3' UTR that is terminated by a poly(A) tract (Tam et al., 1991; Wang et al., 2000). HEV is the causative agent of hepatitis E.

Global HEV strains have been segregated into 4 genotypes, numbered 1–4 (Schlauder and Mushahwar, 2001). Genotypes 1

Abbreviations: HEV, hepatitis E virus; MCMC, Markov chain Monte Carlo; BF, Bayes factor; TMRCA, time of the most recent common ancestor.

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and 2 of HEV infect only humans and are isolated from patients of hepatitis E outbreaks in developing countries, where water sources are sometimes contaminated by faeces containing HEV (Bradly et al., 1993; Okamoto, 2007). In contrast, genotype 3 of HEV has been isolated from autochthonous hepatitis cases all over the world, and genotype 4 of HEV has been isolated from sporadic hepatitis cases mainly in Asian countries such as Japan and China (Abe et al., 2006; Kamar et al., 2012; Lu et al., 2006; Okamoto, 2007). Some patients contract the disease after consumption of meat or offal of swine, wild boar, and deer (Bouwknegt et al., 2007; Reuter et al., 2009; Tamada et al., 2004; Tei et al., 2004; Yazaki et al., 2003). Domestic swine and some wild animals are known as reservoirs of HEV genotypes 3 and 4 (Kamar et al., 2012; Okamoto, 2007). Recently, candidates for new genotypes were reported in wild boars in Japan (Takahashi et al., 2010, 2011).

Lu et al. proposed further subtyping of each genotype through phylogenetic analyses and designated 10 subtypes for genotype 3 of HEV worldwide alphabetically: 3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, 3i,

and 3j (2006). In Japan, polyphyletic HEV strains of genotype 3 have been isolated not only from humans and domestic pigs but also from boars, deer, and mongooses captured in the wild (Li et al., 2005; Nakamura et al., 2006a,b; Nishizawa et al., 2005; Sonoda et al., 2004; Takahashi et al., 2004; Tanaka et al., 2006; Tei et al., 2004). Japan-indigenous HEV genotype 3 isolates were provisionally classified into 3 phylogenetic clusters, i.e., III_{jp}, III_{us}, and III_{sp} (Takahashi et al., 2003b), which corresponded to subtype 3b, 3a, and 3e, respectively (Okamoto, 2007). We previously investigated the molecular evolution and epidemic history of the 3 subtypes by phylogenetic and coalescent analyses (Nakano et al., 2012b). We showed that the III_{jp}/3b (hereafter referred to as 3b) cluster consisted mostly of Japanese isolates, the Japanese III_{us}/3a (hereafter referred to as 3a) isolates were related to 3a isolates from New World countries, and the Japanese III_{sp}/3e (hereafter referred to as 3e) isolates were related to isolates from European countries by phylogenetic tree analysis. We also inferred that isolates of 3 subtypes entered Japan via importation of pigs from European countries or New World countries at different times in the past by coalescent analyses.

HEV infection in wild boars has been investigated in many prefectures in Japan. Many HEV isolates infecting Japanese wild boars have been isolated, and the nucleotide sequences have been characterised (Michitaka et al., 2007; Nakamura et al., 2006a; Nishizawa et al., 2005; Sakano et al., 2009; Sato et al., 2011; Sonoda et al., 2004; Takahashi et al., 2010, 2011). For instance, Sato et al. investigated HEV infection and HEV genomes among 578 Japanese wild boars captured in 25 of 47 prefectures in Japan (2011). HEV infection of wild boars in Mie prefecture, however, has not yet been assessed. Mie prefecture is located in the central region of Japan and is far from the most prevalent regions of HEV infection, which most frequently occurs in the north-eastern part of Japan (Abe et al., 2006; Inoue et al., 2009b; Sainokami et al., 2004). We reported 8 sporadic acute hepatitis E cases caused by almost identical subtype 3e isolates between 2004 and 2011 in Mie prefecture (Nakano et al., 2012a). Although the patients in the 8 cases did not have any common risk factors for HEV infection, such as consumption of uncooked or undercooked meat of wild mammals (boar or deer), we were interested in HEV isolates infecting wild boars in Mie prefecture.

In the present study, we characterised the nucleotide sequence of HEV isolates infecting wild boars in Mie prefecture by phylogenetic and coalescent analyses. We identified subtype 3e isolates from Japanese wild boars in Mie prefecture. This is the first report to confirm the indigenization of subtype 3e isolates in Japanese wild boars, although some subtype 3e isolates in Japan have been reported in acute hepatitis patients (Inoue et al., 2006a, 2009a; Mizuo et al., 2005; Nakano et al., 2012a) and blood donors (Fukuda et al., 2007), and in swine (Okamoto et al., 2001; Sapsutthipas et al., 2009; Takahashi et al., 2003b; Urayama et al., 2010). We further discuss the meaning of the indigenization of subtype 3e isolates in wild boars living outside Europe, although subtype 3e isolates are usually detected in European countries (Widen et al., 2011).

2. Material and methods

2.1. Detection and determination of nucleotide sequences of the partial ORF1 or ORF2 region of HEV RNA in serum samples of wild boars captured in Mie prefecture

Serum samples were obtained from a total of 144 wild boars (*Sus scrofa*) that had been captured in Mie prefecture between 2008 and 2012. The samples were stored at -20°C or lower temperature until detection and nucleotide sequencing of HEV-RNA. Detection of HEV-RNA and determination of partial 326 nt of the

ORF1 region (nucleotide number 125–450: M73218) or 412 nt of the ORF2 region (nucleotide number 5944–6355) were performed according to a method reported previously (Okamoto et al., 2001; Takahashi et al., 2001). These two regions had been thoroughly sequenced and frequently used for phylogenetic analyses of Japanese isolates. The final products were sequenced in a 3100 DNA Sequencer using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

2.2. Determination of the near-complete nucleotide sequences of 4 HEV isolates

The near-complete nucleotide sequences of 4 subtype 3e isolates from the wild boars were determined by a previously reported method (Takahashi et al., 2001, 2003a). In brief, 23 overlapping regions of the HEV genome were amplified from the cDNA using primers specific for each region. The sequences enriched in G–C were amplified with the TaKaRa LA Taq in GC Buffer (TaKaRa Shuzo Co. Ltd., Shiga, Japan). The sequences not amplifiable by the above PCR methods were subjected to PCR with primers deduced from adjacent 5' and 3' sequences. The final products were sequenced by the same method used for the partial ORF1 or ORF2 region.

2.3. Data set of entire or near-complete nucleotide sequences

Sixty-three entire or near-complete nucleotide sequences were downloaded and selected for genotype 3 HEV from the Hepatitis Virus Database (<http://s2as02.genes.nig.ac.jp/index.html>). These sequences and the 4 HEV subtype 3e sequences determined in this study were compared and subjected to phylogenetic analyses.

2.4. Short ORF2 region data sets

All available nucleotide sequences of the 301-nt ORF2 (nucleotide number 5994–6294) were downloaded from the Hepatitis Virus Database using a map viewer, and useful sequences were selected as we reported previously (Nakano et al., 2012a). This region has been thoroughly sequenced and frequently used for genotyping and subtyping since Mizuo et al. originally compared Japanese HEV isolates with global HEV isolates (2002). Lu et al. also used this region when genotyping and subtyping global HEV isolates by phylogenetic analyses (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. Only genotype 3 sequences were retained. As described in detail in the Section 3, most of the isolates in the present study were closely related to the 3e isolates. For more detailed phylogenetic analyses to address the relationship between our 3e isolates and global 3e-related isolates, we collected more 3e-related isolates through a BLAST search at DDBJ (<http://www.ddbj.nig.ac.jp/Welcome-j.html>) by applying the ORF2-301-nt fragment of our 3e isolates, JBOAR111-Mie11 and JBOAR012-Mie08, as a query sequence. As a result, more sequences that had a homology of >90% with the query sequence were added for the short ORF2 data sets. The resulting short ORF2 alignment contained 245 Japanese genotype 3 sequences, including 7 sequences of the present study and 89 foreign genotype 3 sequences.

2.5. Japanese wild animal HEV and wild animal-related HEV datasets

HEV sequences isolated from wild animals and HEV isolates obtained from acute hepatitis patients who had consumed the meat of wild animals were collected from Japanese studies and case reports. Sixteen entire or near-complete nucleotide sequences isolated from human, wild boar, wild deer, and wild mongoose,

including the 4 sequences in this study, were included in these datasets (Inoue et al., 2006a; Kawamura et al., 2010; Nakamura et al., 2006b; Nishizawa et al., 2005; Okita et al., 2012; Sato et al., 2011; Takahashi et al., 2004, 2010, 2011). For the analysis of the 326-nt ORF1 region (nucleotide number 125–450), 25 sequences, including 1 sequence in this study, were added (Hijioka et al., 2005; Masuda et al., 2005; Michitaka et al., 2007; Nakamura et al., 2006a; Sakano et al., 2009; Sato et al., 2011; Tamada et al., 2004). For the analysis of the 412-nt ORF2 region (nucleotide number 5944–6355), 24 sequences, including 3 sequences in this study, were added (Hijioka et al., 2005; Li et al., 2005; Nakamura et al., 2006a; Nidaira et al., 2012; Sato et al., 2011).

2.6. Phylogenetic tree analyses

We constructed neighbour-joining trees and maximum likelihood (ML) phylogenies to evaluate the phylogenetic clustering of the nearly complete ORF, 301 nt of ORF2, 412 nt of ORF2, and 326 nt of ORF1 data sets by using SeaView version 4 (Gouy et al., 2010). ML phylogenies were heuristically searched using subtree pruning and regrafting as well as nearest neighbour interchange perturbation algorithms. The statistical robustness of the phylogenetic groupings was subsequently assessed using bootstrap analyses (1000 replicates for neighbour-joining trees and 100 replicates for ML phylogenies). Phylogeographic structure was then identified using FigTree (available from <http://tree.bio.ed.ac.uk>).

2.7. Coalescent analyses of Japan-indigenous genotype 3 HEV isolates

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), using a method reported previously (Nakano et al., 2012a), with some modifications. In brief, we analysed 412 nt of the ORF2 region (nucleotide number 5944–6355) of Japan-indigenous genotype 3 isolates with known sampling dates. In total, 199 sequences, including the seven sequences in the present study, were analysed, and 14 subtype 3e sequences, including six sequences in this study, were added to the previous analyses to reconfirm the time of the most recent common ancestors (TMRCA) of Japan-indigenous subtype 3e. Markov chain Monte Carlo (MCMC) sampling was performed for at least 2×10^8 generations, and a tree was sampled every 10,000 generations. We analysed 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. We estimated Bayes factors (BFs) to choose the most statistically appropriate combination of clock model and demographic model (Suchard et al., 2001). The program Tracer (<http://tree.bio.ed.ac.uk>) was used to estimate BFs, check for convergence, and 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. The program TreeAnnotator (Drummond and Rambaut, 2007) was used to construct a phylogeny that best summarized the set of credible trees, called the maximum clade support phylogeny. In this Bayesian MCMC analysis, the branch lengths and node heights of the maximum clade support phylogeny are in units of years (Drummond et al., 2006). The phylogeographic structure was then identified using FigTree.

3. Results

3.1. Detection and nucleotide sequence analyses of HEV RNA among wild boars in Mie prefecture

Among 144 serum samples of wild boars captured in Mie prefecture, 5 were positive for the 326-nt ORF1 region of HEV-RNA (JBOAR050-Mie09, JBOAR100-Mie10, JBOAR107-Mie11, JBOAR111-Mie11, JBOAR124-Mie11). These 5 samples and 2 additional samples (JBOAR012-Mie08, JBOAR120-Mie11) were positive for the 412-nt ORF2 region of HEV-RNA. Among the 7 positive samples, the nucleotide sequence of nearly the entire genome could be determined for 4 samples.

In the neighbour-joining tree of full ORF region sequences, 67 genotype 3 isolates, including isolates identified in the present study (Mie boar isolates), were tentatively divided into subtypes 3a, 3b, 3e, 3f, 3g, and 3j, as well as ambiguous or undetermined sequences (Fig. 1). Significant bootstrap support was obtained for each subtype. Similar results were obtained in the maximum likelihood (ML) tree (data not shown). The 4 Mie boar isolates were closely related to each other and clustered with 3e isolates as guides with a bootstrap value of 100%. The simple homology among the 4 sequences was 99.7–99.8%. The isolates most related to the 4 Mie boar isolates in the tree were HE-JA04-1911 and JNH-Ehi04L, which were recovered from acute hepatitis patients who contracted the disease in the same prefecture, Mie (Inoue et al., 2006b), and in a distant prefecture, Ehime (Inoue et al., 2006a). However, the homology between the 4 Mie boar sequences and the 2 isolates was 95.9–96.1%, which did not indicate an intimate relationship. The phylogenetic relationship between the 4 Mie boar isolates and swine isolates swj8-5 and swj12-4 (Inoue et al., 2006b) from a remote region in Japan was even more distant. The phylogenetic relationship between the 4 Mie boar isolates and swine isolates from Hungary, i.e. HEV072/sw/HUN-05 (Forgach et al., 2010), or a boar isolate from Germany, i.e. SA21 (Adlhoch et al., 2009), was also distant. Because of the shortage of full-genome sequences of subtype 3e isolates from European countries, the relationship between the Mie boar isolates and European isolates could not be sufficiently assessed, although the 4 Mie boar isolates were obviously judged as subtype 3e.

Fig. 2 shows the neighbour-joining tree of the 301-nt sequence within the ORF2 region. According to the increasing number of sequences, the structure of the subtyping 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 observed in the small phylogeny based on the corresponding full ORF sequences. Subsequently, 334 genotype 3 isolates were tentatively divided into subtypes 3a, 3b, 3c, 3d, 3e, 3f, 3g, and undetermined. A 3j isolate (AY115488) from Canada was included in the 3b cluster in this tree, possibly because of the weak phylogenetic signal of the short region (Fig. 2b). The 3b cluster consisted mostly of Japanese isolates (Fig. 2b), and the Japanese 3a isolates were separated into many lineages by many groups of isolates from Western countries and Korea as we reported previously (Fig. 2c) (Nakano et al., 2012b). A Mie boar isolate (JBOAR050-Mie09) was included in the 3a cluster. The origins of the 3b cluster and Japanese 3a clusters were ambiguous from the structure of the tree. Thirty-seven isolates out of 245 Japanese isolates belonged to the 3e cluster (Fig. 2a). The 37 Japanese 3e isolates were further separated into several lineages. Six Mie boar isolates were included in subtype 3e. JBOAR012-Mie08 was located separately from the other 5 isolates. The 5 isolates, 4 of which were mostly identical in the full ORF sequence analysis, were closely related and

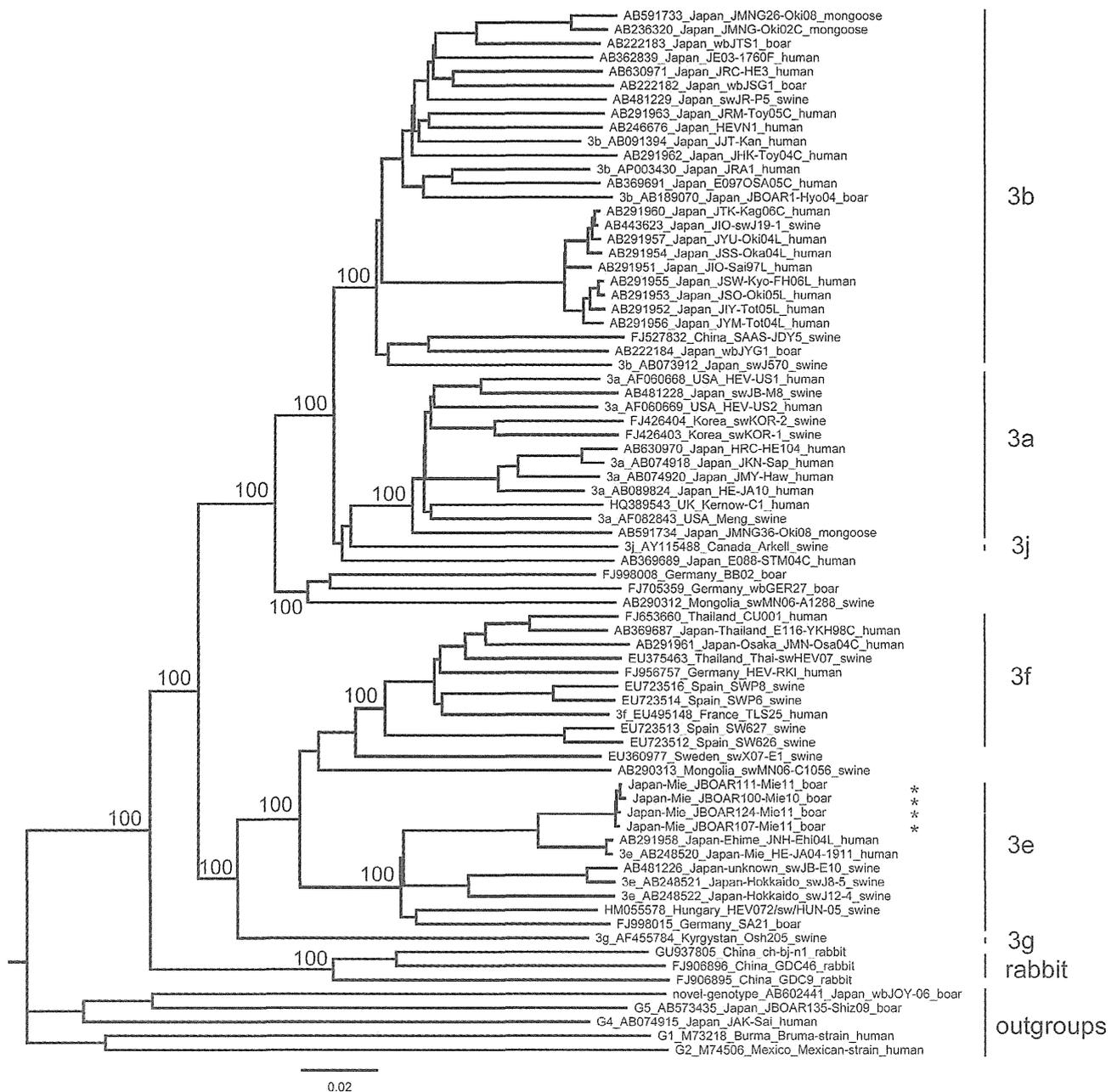


Fig. 1. Phylogenetic tree constructed by the neighbour-joining method based on the full ORF nucleotide sequences of 67 HEV genotype 3 isolates, using three rabbit and five other genotype sequences as outgroup sequences. Sequences in the previous literature are included as guides for subtyping (Inoue et al., 2006b; Lu et al., 2006). Genotypes and subtypes of the sequences are shown at the top of the sequence names. Accession numbers, location of isolation, name of isolates, and host are indicated in sequence names. The Mie boar sequences are indicated by "*" following the sequence name. The scale bar indicates nucleotide substitutions-site⁻¹. Bootstrap values are indicated as a percentage for the major nodes. Subtypes of clusters are indicated at the right of the tree. Subtype is not indicated for isolates whose subtype was ambiguous or undetermined.

clustered together with 93% bootstrap value. The 5 isolates also clustered together with 9 human or swine isolates from a wide area of Japan, i.e., Mie, Ehime, Miyagi, and Tochigi prefectures, with a bootstrap value of 94%. The 14 isolates were nested under the French isolate TLS38 (EU495229) (Legrand-Abravanel et al., 2009). The bootstrap value of 77% firmly supported clustering of the 14 Japanese isolates and the French isolate. Isolates from other European countries were present upstream of the French isolate. The phylogenetic structure strongly suggests that the ancestor of the 5 Mie boar isolates originated in Europe. An isolate recovered from wild boar in Thailand, i.e. VSMU-HEV-10 (Wiratsudakul et al., 2012), which was reported as subtype 3e, was suggested as subtype 3f in our phylogenetic tree. A Mongolian isolate,

swMN06-C1056 (AB290313) (Lorenzo et al., 2007), which was situated between 3e and 3f in the full ORF tree, was temporally clustered in the 3e cluster in the 301-nt ORF2 region tree.

3.2. HEV genotype and subtype infecting Japanese wild animals

The phylogenetic tree of the 326-nt ORF1 region was constructed from 41 HEV sequences isolated from wild animals captured in Japan and obtained from acute hepatitis patients who had consumed the meat of wild animals captured in Japan (Fig. 3a). The phylogenetic tree of the 412-nt ORF2 region was also constructed from 40 sequences (Fig. 3b). Forty-one or 40 isolates were divided into 3a, 3b, 3e, genotype 4, and novel genotypes in

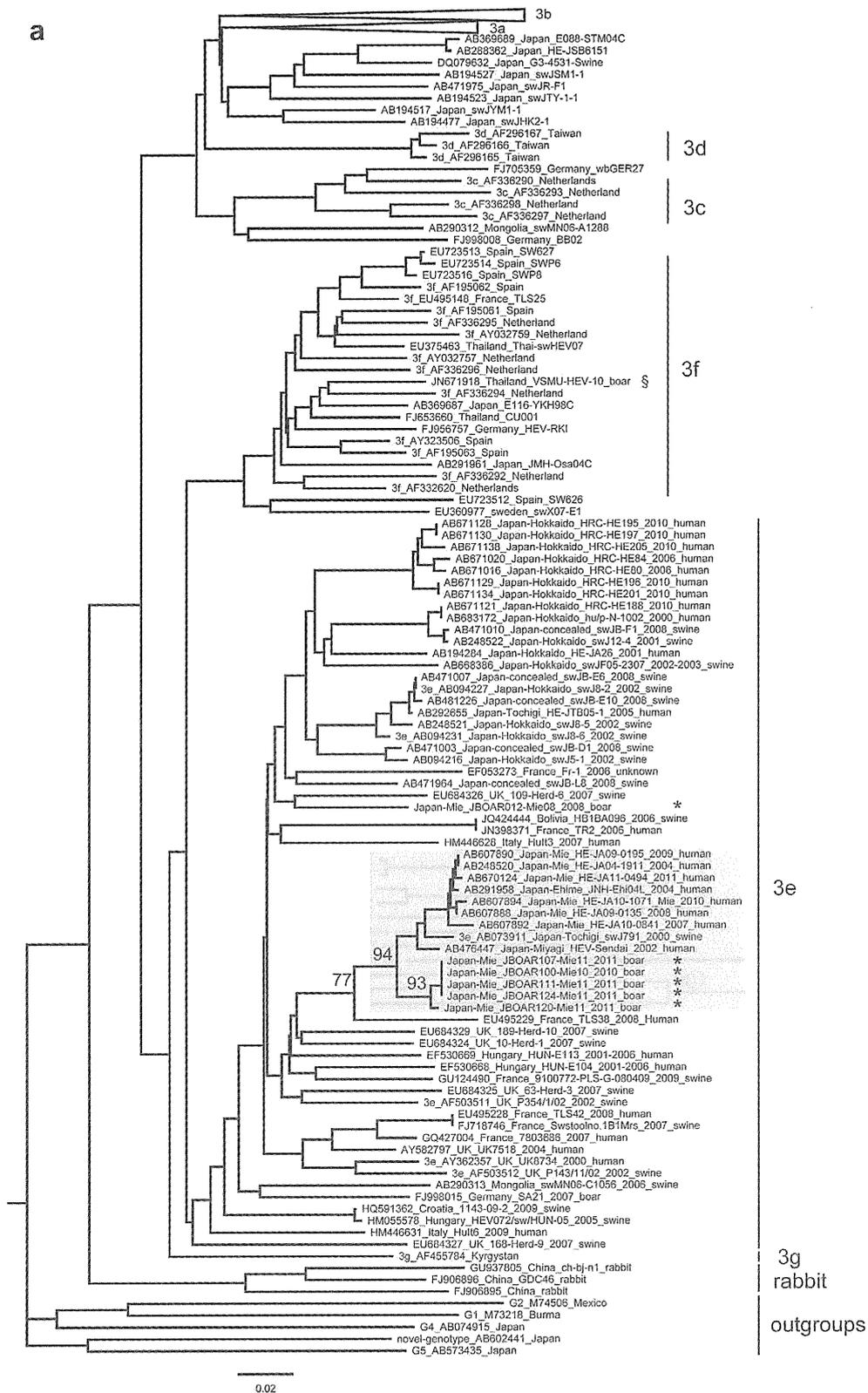


Fig. 2. The neighbour-joining tree of the 301-nt ORF2 alignment containing 334 genotype 3 global isolates with 3 rabbit and 5 other genotype sequences as outgroup sequences. Because it was difficult to indicate all isolates in one figure given the large number of sequences, clusters of subtypes 3b and 3a clusters are collapsed in (a) and are shown in (b) and (c), respectively. Clusters of other subtypes and outgroup sequences are shown in (a). Genotypes and subtypes are shown at the top of the sequence names of the representative isolates of each type. Accession numbers, location of isolation, name of isolate, and host are indicated in sequence names. For subtype 3e isolates, the year of isolation is also indicated. The Mie boar sequences are indicated by "*" following the sequence name. The scale bars indicate nucleotide substitutions site⁻¹. Subtypes of clusters are indicated at the right of the tree. Subtype is not indicated for isolates whose subtype was ambiguous or undetermined. Bootstrap values are indicated for a node for 5 closely related Mie boar isolates, for a node for 14 Japanese subtype 3e isolates (shaded area), and for a node that connects the Japanese subtype 3e cluster in the shaded area with the French isolate TLS38. An isolate recovered from a wild boar in Thailand, VSMU-HEV-10, is indicated by "\$." A 3j isolate (Y115488) from Canada is indicated by "#."



Fig. 2 (continued)

the same manner in both trees. The Mie boar isolates, i.e. 5 in the ORF1 tree and 7 in the ORF2 tree, were divided and clustered as they were in the trees containing the global full ORF (Fig. 1) or the short 301-nt of ORF2 region (Fig. 2). A Mie boar isolate, JBOAR050-Mie09, was included in subtype 3a. Four and 6 Mie boar

isolates were included in subtype 3e in each tree. Five Mie boar 3e isolates were closely related. One Mie boar 3e isolate, JBOAR012-Mie08, was positioned separately. Based on the above phylogenetic analyses, the distribution of genotypes and subtypes of Japanese wild animal HEV isolates and wild animal-related HEV isolates is

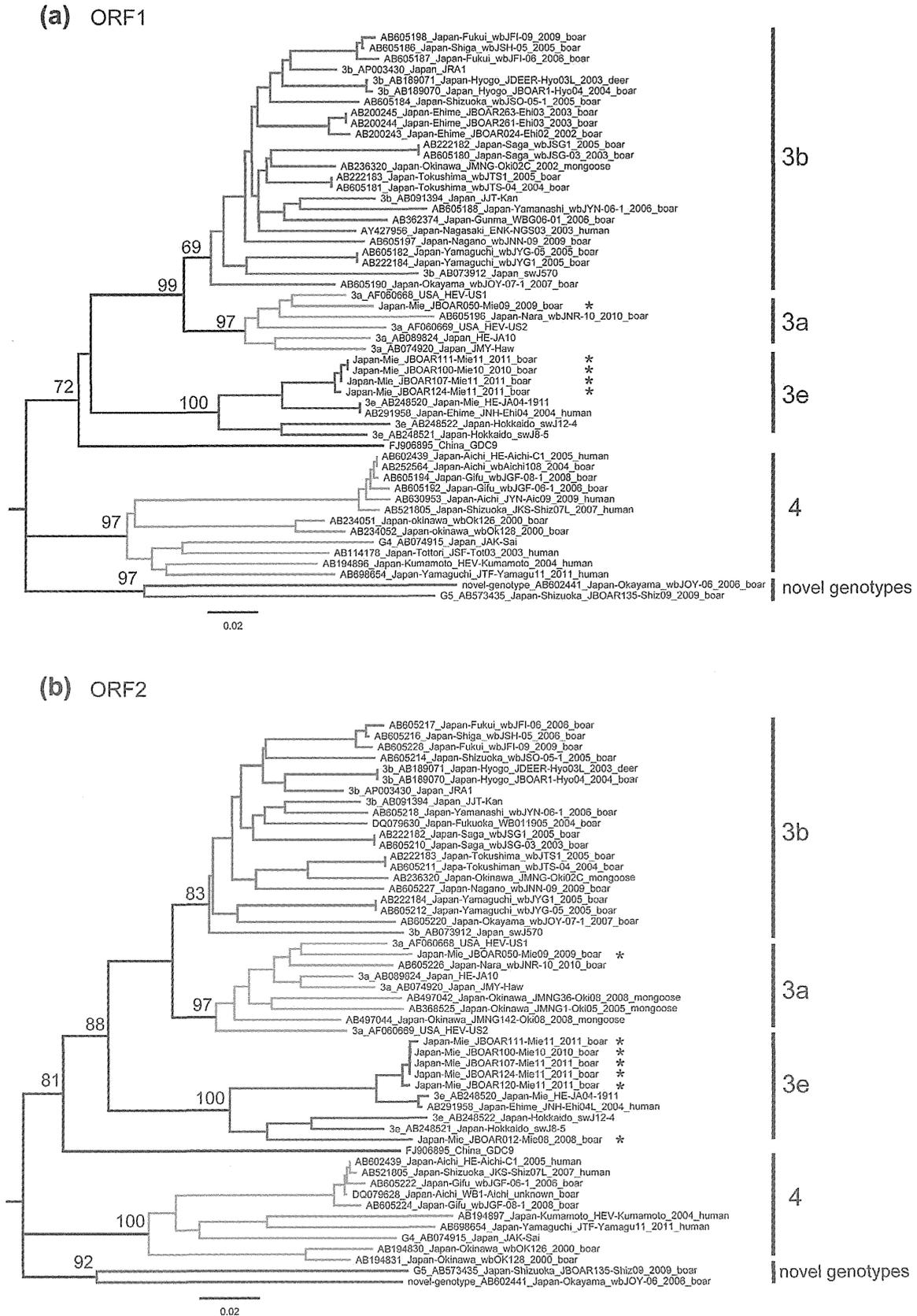


Fig. 3. The phylogenetic tree of 326-nt ORF1 (a) and 412-nt ORF2 (b) regions constructed from HEV sequences isolated from wild animals captured in Japan or HEV sequences obtained from acute hepatitis patients who had consumed the meat of wild animals captured in Japan. Genotypes and subtypes are shown at the top of the sequence names of the representative isolates of each subtype. Accession numbers, location of isolation, name of isolates, year of isolation, and host are indicated in the sequence names. In both of the trees, branches of each genotype and subtype are coloured as follows: 3b, red; 3a, green; 3e, blue; 4, orange; and novel genotypes, grey. The Mie boar sequences are indicated by “*” following the sequence name. The scale bars indicate nucleotide substitutions/site⁻¹. Bootstrap values are indicated for the major nodes as a percentage. The rabbit sequence isolated in China was also included for comparison (Zhao et al., 2009). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

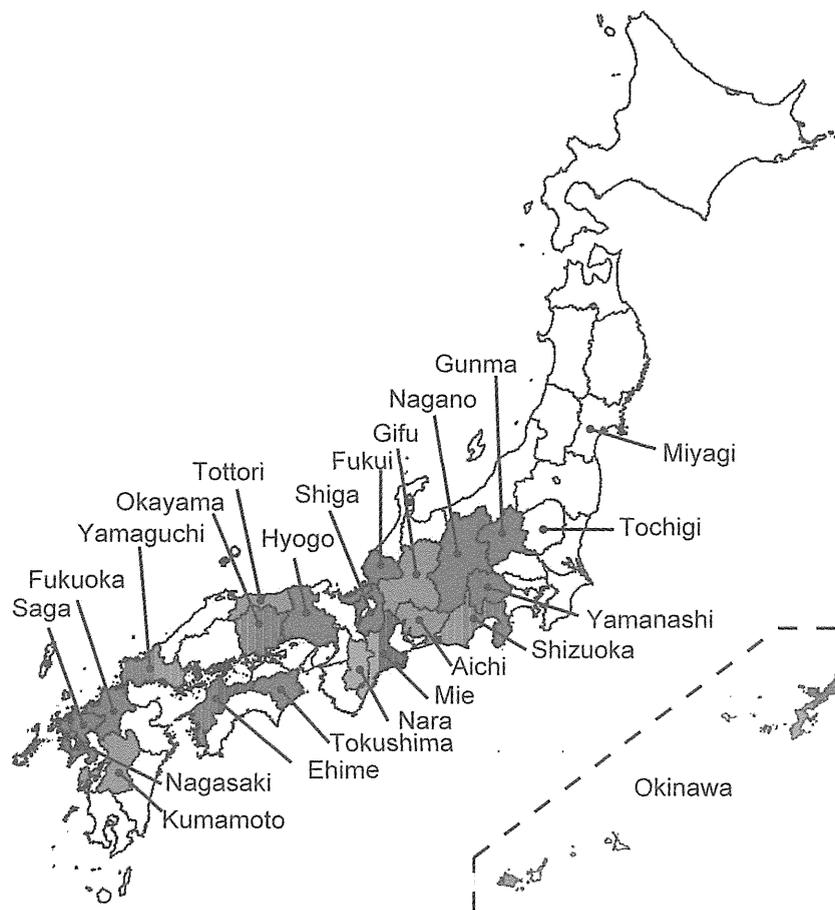


Fig. 4. Distribution of HEV genotypes and subtypes among wild animals in Japan. Prefectures where HEV sequences were isolated from wild animals or where HEV isolates were obtained from acute hepatitis patients who had consumed the meat of wild animals are indicated by colours. Each prefecture is coloured according to the respective isolated HEV genotypes and subtypes as described in Fig. 3. When multiple genotypes and subtypes were isolated in 1 prefecture, the prefecture was indicated by multiple colours (i.e., Mie prefecture was indicated in blue and green [3e and 3a in this study]). Miyagi and Tochigi prefectures are also indicated for the following results in Fig. 5 although HEV isolates have not been reported from wild animals in those prefectures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

shown in Fig. 4. Subtype 3b isolates were found in 14 prefectures, including Gunma, Nagano, Yamanashi, Shizuoka, Fukui, Shiga, Hyogo, Okayama, Yamaguchi, Tokushima, Ehime, Fukuoka, Saga, and Okinawa. Genotype 4 isolates were prevalent in seven prefectures, including Shizuoka, Gifu, Aichi, Tottori, Yamaguchi, Kumamoto, and Okinawa. Subtype 3a isolates were rare and recovered from only three prefectures: Mie, Nara, and Okinawa. Subtype 3e isolates were also rare and isolated from only two prefectures: Mie and Ehime.

3.3. Estimation of the TMRCA of Japan-indigenous subtype 3e and Mie boar subtype 3e isolates

Coalescent analyses of the dated 412-nt ORF2 region data set were performed in BEAST under various molecular clock and coalescent model combinations. The combination of an uncorrelated exponential clock model and Bayesian skyline yielded the best estimates of evolutionary rate and TMRCA. The evolutionary rate was 1.59×10^{-3} substitutions-site⁻¹ year⁻¹ (95% credible interval [CI], 1.09×10^{-3} to 2.11×10^{-3}). The TMRCA were calculated based on the clustering of the maximum clade support phylogeny (Fig. 5). The TMRCA for all Japanese subtype 3e isolates was 1966 (95% CI, 1942–1978), which was very similar to our previous results (Nakano et al., 2012a), although more subtype 3e sequences, including 5 sequences in this study, were newly added as dated

subtype 3e sequences. The TMRCA for 14 isolates, including the 5 Mie boar and 9 other human or swine isolates from a wide area of Japan, was 1992 (95% CI, 1979–2000). The TMRCA for the 5 Mie boar isolates was 2009 (95% CI, 2006–2011).

In the maximum clade support phylogeny, all Japanese subtype 3e isolates; 14 subtype 3e isolates, including the 5 Mie boar isolates; and the 5 Mie boar 3e isolates clustered together with high posterior probabilities (equal to 1). A cluster of all Japanese subtype 3e isolates branched from the other subtypes at the oldest age with no more branching until after 1960. The branch of the 5 Mie boar isolates and other 9 subtype 3e isolates diverged around 1990, and the branch of the 5 Mie boar isolates then started to diverge around 2010, which corresponded to the values of the TMRCA.

4. Discussion

The classification of HEV variants is currently in transition without agreed definitions for genotypes and subtypes. New grouping methods have been proposed but they have not been accepted universally yet (Bouquet et al., 2012; Oliveira-Filho et al., 2013). A more comprehensive grouping method introduced by Lu et al. (2006) is still used by many researchers although problems of this method have been pointed out (Smith et al., 2013). We tentatively tried to elucidate the relationship between the Mie boar isolates

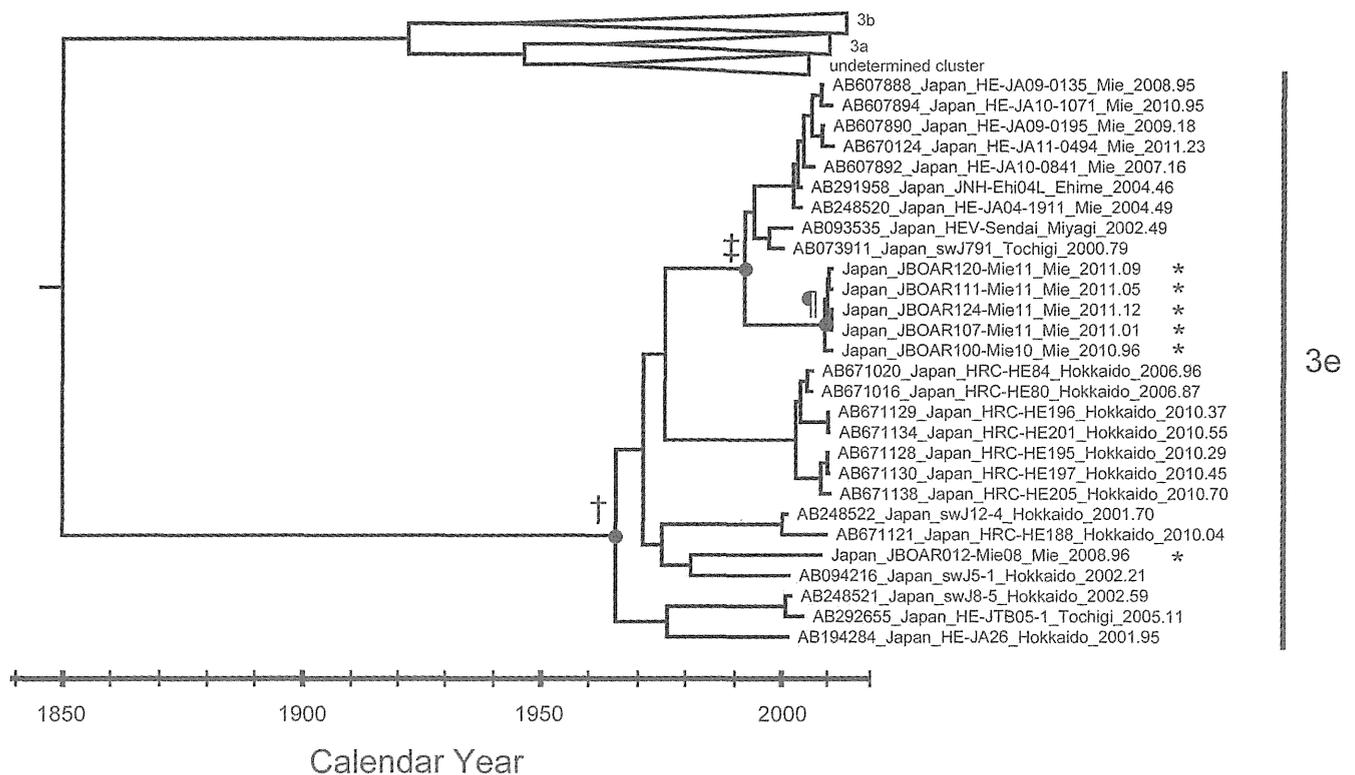


Fig. 5. The maximum clade support phylogeny for the dated Japanese sequence set of the ORF2 region (412 nt). Subtype 3b, 3a, and undetermined clusters are collapsed, and the detail is shown in Supplemental Fig. 1. Accession numbers, isolated country, name of isolates, isolated prefecture, and isolated date are indicated in sequence names. The node with “†” unifies all of the Japanese 3e strains. The node with “●” merges 14 isolates, including the 5 Mie boar isolates and 9 other human or swine isolates. The node with “◻” integrates the 5 Mie boar isolates in this study. The Mie boar isolates are indicated by “*” following the names of isolates. The branch lengths and node heights are in units of years.

and the global genotype 3 isolates based on the grouping method of Lu et al. Most sequences reported from various countries are relatively short, although analysis of longer or even whole-genome sequences should be more reliable for accurate identification of genetic relatedness among HEV isolates. Especially for European isolates, longer or full-genome sequences are so limited that analysis of a short region was unavoidable. Thus, we analysed the sequences of 301-nt of the ORF2 region using phylogenetic analysis (Fig. 2). As more sequences were added, the subtyping became more ambiguous, and clusters for each subtype were not supported by bootstrap analyses. The use of short sequences introduced the possibility of ambiguous or discordant subtyping because of the weak phylogenetic signal of the short region (i.e. 3j isolate: AY115488, a Mongolian isolate, swMN06-C1056: AB290313). Genetic similarity within a short genomic region should therefore be interpreted with utmost caution when identifying genetic relatedness among viral isolates. Analysis of an insufficient number of reference sequences may also lead to incorrect subtyping (i.e. a Thailand isolate, VSMU-HEV-10: JN671918). Analysis using sufficient reference sequences of extended genomic regions or the entire genome should be considered for a reliable genetic characterization of HEV isolates. In the present study, we also analysed the sequences of the full ORF region, but an insufficient amount of available reference sequences prevented us from determining the origin of Mie boar isolates. Determination and submission of longer sequences of HEV isolates are anticipated, especially for European isolates.

Based on our phylogenetic analyses (Fig. 3), subtype 3b and genotype 4 isolates seemed prevalent among wild animals in many prefectures in Japan (Fig. 4). Subtype 3a and 3e isolates were rare. One 3a isolate was recovered from Mie prefecture in the present

study, and another isolate was reported from a neighbouring prefecture, Nara (Fig. 4) (Sato et al., 2011). The other 3a isolates were found in mongooses captured in Okinawa, which is far from Mie and Nara prefectures (Nidaira et al., 2012). In the present study, 6 subtype 3e isolates were recovered from wild boars in Mie prefecture. Another 3e isolate from Ehime prefecture was recovered from an acute hepatitis patient who had a history of handling, cooking, and eating wild boar meat 1 month before the onset of her disease (Inoue et al., 2006a). However, in that study, the subtype 3e isolate was not directly recovered from wild boar meat. It is intriguing that all of our seven Mie boar isolates were of the rare 3a or 3e subtypes. In particular, our six subtype 3e isolates were the first subtype 3e isolates to be recovered from wild animals in Japan; five isolates were closely related, and four out of the five isolates were sequenced almost entirely. We confirmed that subtype 3e isolates were indigenized in wild boars in Mie prefecture.

The phylogenetic analyses indicated that subtype 3b was peculiar to Japan and was the most major subtype in Japan (Figs. 1 and 2b). Its origin or specific relationship with isolates from other countries was not distinguished by the structure of the trees. In the 3a cluster, many groups of Japanese isolates were intricately intertwined with many groups of isolates from Western countries and Korea (Fig. 2c). The presence of multiple old phylogenetic nodes that connected Japanese clusters and lineages from other countries suggests that the historical inflow of 3a isolates occurred several times in the past. However, the origin or direction of the inflow or dissemination was not clear from the structure of the trees (Figs. 1 and 2c). In the 3e cluster, lineages of Japanese isolates were intricately intertwined with those of isolates from European countries (Fig. 2a). The presence of old phylogenetic nodes that

connected Japanese lineages and the lineages of isolates from European countries suggests that historical inflow of 3e isolates occurred several times in the past. One of the lineages that included 5 Mie boar isolates was clearly nested within the French 3e isolate, TLS38: EU495229, as supported by a sufficient bootstrap value rather than the weak phylogenetic signal of the short region. The phylogenetic structure supported the direction of historical inflow of subtype 3e isolates from Europe to Japan. Subtype 3e isolates were obtained from acute hepatitis patients, domestic swine, and wild animals, mainly in European countries such as the UK (Banks et al., 2004), France (Colson et al., 2010; Kaba et al., 2009; Rose et al., 2011), the Netherlands (Rutjes et al., 2009), Germany (Adlhoch et al., 2009; Wichmann et al., 2008), Hungary (Forgach et al., 2010; Reuter et al., 2009), Sweden (Widen et al., 2011), and Slovenia (Steyer et al., 2011). The present report is the first report of indigenization of HEV subtype 3e isolates among wild animals outside Europe. It is unlikely that wild mammals (i.e., wild boars) invaded Japan by themselves as an HEV reservoir, because Japan is far from Europe and is surrounded by an ocean and a sea. Recently, we reported that the import of a breed of large-race pigs from Europe since the 1960s may be responsible for the introduction of subtype 3e isolates to Japan (Nakano et al., 2012a,b). Although we added more sequences of Japanese subtype 3e isolates for the calculation of TMRCA in the present study, the result did not differ from that of previous studies. The confirmed TMRCA, 1966, suggests that the ancestor of Japan-indigenous subtype 3e originated in Europe; particularly, the ancestor of subtype 3e in Japan entered Japan from European countries through the importation of large-race pigs in the 1960s.

Transmission of HEV genotype 3 or 4 from swine to wild boars (or the reverse) is not unexpected. However, in fact, there is no evidence of direct transmission between swine and wild boars. There is no report that HEV sequences isolated from swine and wild boars have close similarity (probably more than 99%) sufficient to indicate direct transmission. We also could not obtain direct evidence of HEV transmission from swine to boars. Rather, we provide indirect evidence of swine-to-boar transmission of subtype 3e by gene flow as indicated by molecular evolutionary analyses. The phylogenetic and coalescent analyses in the present study led us to consider the following scenario of dissemination of subtype 3e isolates in Japan. Subtype 3e of European origin entered Japan through the importation of pigs. Then, subtype 3e probably expanded in swine after 1966. One of the lineages showed division into several lineages, including those giving rise to the five Mie boar and nine other human or swine isolates around 1992, which were detected across Japan. One of these lineages gave rise to the cluster of Mie boar isolates. The TMRCA of the five Mie boar isolates was around 2009. Between 1992 and 2009, the cluster of Mie wild boar isolates appeared in the peripheral branch of the phylogenetic lineage. It may indicate the direction of gene flow of HEV subtype 3e from swine to wild boars. Our analyses may thus provide indirect evidence of transmission of HEV from swine to wild boar in the past. Indigenization of subtype 3e isolates in the Japanese wild boar may suggest subtype 3e transmission from swine to wild boar. Investigation of HEV infection in pig farms of the same region may shed light on the origin of the Mie boar isolates although inspection of HEV infection in hog factories is difficult because of the fear of damage caused by rumours.

The above assumption does not exclude the reverse direction of HEV transmission, i.e. from wild boar to swine. In the case of subtype 3e in Europe, there is no direct evidence of transmission between swine and wild boars. Genotype 3b isolates, which were found mainly in Japan, were typically from both swine and wild animals (Sato et al., 2011; Takahashi et al., 2003b). Genotype 4 isolates were found mainly in Asia and were typically from both swine and wild animals (Liu et al., 2012; Sato et al., 2011;

Takahashi et al., 2003b). Both directions of transmission are possible, and both directions of infection should be considered based on the extensive spread of genotype 3b in Japan and genotype 4 in Asia in swine and wild animals. For new genotypes isolated from only wild boars (Takahashi et al., 2010, 2011), there have been no reports or evidence of transmission to swine or humans.

5. Conclusions

It is likely that subtype 3e HEV is extensively spread and was originally indigenized in European countries. The phylogenetic and coalescent analyses indicated that subtype 3e HEV was disseminated to Japan from Europe by importation of infected pigs. In the present study, we specifically identified the indigenization of subtype 3e isolates in Japanese wild boars. The phylogenetic and coalescent analyses also suggested the direction of gene flow of HEV subtype 3e from swine to wild boars. This finding may thus provide indirect evidence of transmission of HEV subtype 3e from swine to wild boar in the past in Japan. Clarification of the transmission direction or route of HEV infection should be helpful to prevent a future endemic or epidemic of HEV infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.meegid.2013.06.004>.

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Epidemiological and Molecular Analyses of a Non-Seasonal Outbreak of Acute Icteric Hepatitis E in Bangladesh

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Acute hepatitis due to hepatitis E virus (HEV) is endemic in Bangladesh, but its epidemiological characteristics and virological features remain obscure. An outbreak of acute icteric hepatitis E occurred in Rajshahi, Bangladesh during 2010 when 200 patients with visible jaundice visited physicians within a period of 1 month (January–February). Clinical and epidemiological data were collected from these patients using questionnaires. Nucleic acids were isolated from 15 patients who were selected at random to ascertain their HEV genotypes. Near-complete nucleotide sequences of the HEV genome were detected in two patients and partial ORF2 regions in the other 13 patients. All patients tested positive for IgM antibodies to HEV but negative for other hepatitis viruses. Most patients were icteric and complained of vomiting, fever, itching, and abdominal pain. All 15 HEV sequences formed a single cluster within genotype 1a. Two of the 7,186-nt HEV sequences were 99.8% identical. This is the first study to report the clinical, epidemiological, and molecular characterization of an outbreak of acute hepatitis E in Bangladesh. *J. Med. Virol.* **85:1369–1376, 2013.** © 2013 Wiley Periodicals, Inc.

KEY WORDS: acute icteric hepatitis E; Bangladesh; full genome sequencing; hepatitis E virus, genotype 1

Shrestha et al., 2003]. Bangladesh, a Southeast Asian country with a population of 160 million, does not have a safe water supply, hygienic disposal of sewage, or the correct reporting of communicable diseases. Approximately 25% of the rural residents of Bangladesh express IgG antibodies to HEV in their sera [Labrique et al., 2009]. It has been reported that HEV is the main etiological agent of acute hepatitis [Al-Mahtab et al., 2009] and the single major cause of fulminant hepatitis [Sheikh et al., 2002]. However, there is a paucity of information about the epidemiological characteristics, clinical features, and genomic analysis of acute hepatitis due to HEV in Bangladesh.

An outbreak of HEV-induced acute icteric hepatitis was reported during January and February 2010 in Rajshahi, a Northern district of Bangladesh. Two hundred patients with acute jaundice visited physicians during this period. This is not a usual phenomenon in Bangladesh because endemic or epidemic outbreaks of acute hepatitis E are usually detected during the rainy season in August and September. No outbreaks of HEV were detected in any other regions of Bangladesh at that time. The epidemiology of this outbreak of sporadic acute hepatitis E was investigated by developing and administering a questionnaire to analyze clinical data. The sera from 15 patients with acute hepatitis E were also selected at random to determine their HEV genotypes. Finally, the almost complete nucleotide (nt) sequences of the

INTRODUCTION

Viral hepatitis caused by the hepatitis E virus (HEV) is mainly a waterborne infection in most of the developing countries in Asia and Africa [Balayan et al., 1983; Ray et al., 1991; Ticehurst et al., 1992;

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HEV genomes from two patients with acute hepatitis E that had the highest levels of HEV RNA were determined and a phylogenetic tree was constructed.

MATERIALS AND METHODS

A total of 200 patients with acute icteric hepatitis E visited physicians in Rajshahi over a period of 1 month (January 2010–February 2010). All patients were positive for IgM antibodies to HEV (IgM anti-HEV) but negative for IgM type antibodies to hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis C virus (HCV). Informed consent was obtained from all patients after explaining the nature and purpose of the study. The study protocol was also approved by the review board at the Viral Hepatitis Foundation of Bangladesh, Dhaka, Bangladesh. A questionnaire was prepared for patients to collect information about their age, gender, occupation, source of drinking water, and place of habitation (rural or urban). Additional information was obtained about each patient's history of anorexia, vomiting, diarrhea, pruritis, abdominal pain, and weight loss.

Assessment of Serological and Biochemical Markers

IgM antibodies to HEV, HAV, HBV, and HCV were measured using commercial kits (Atlas Medical, Cambridge, UK). The levels of alanine aminotransferase (ALT) and bilirubin in the sera were measured using commercial kits. The hepatitis B surface antigen (HBsAg) and antibodies to HCV (anti-HCV) were measured using an enzyme-linked immunosorbent assay. Ultrasonography was performed in all patients to assess whether they were suffering from chronic liver diseases or complications, such as liver cirrhosis or hepatocellular carcinoma.

RNA Extraction, cDNA Synthesis, Amplification, and Sequencing

Isolation of RNA, genotyping of HEV, and near-complete nt sequencing of HEV genomes were performed according to previously described methods, with some modifications [Takahashi et al., 2009]. Nucleic acids were extracted from sera using a QIAamp MinElute Virus Spin Kit (Qiagen GmbH, Hilden, Germany). HEV RNA genomes were reverse transcribed and cDNA was amplified using PCR with primers specific for 23 overlapping regions in the HEV genome (Table I). The reverse transcription and first-round PCR were conducted using a PrimeScript II High Fidelity One Step RT-PCR Kit (Takara Bio, Inc., Shiga, Japan), while the second-round PCR was conducted using PrimeSTAR GXL DNA Polymerase (Takara Bio, Inc.). A 3'-Full RACE Core set (Takara Bio, Inc.) was used to amplify the core 3' sequences. The final products were sequenced using a 3,100 DNA sequencer with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City,

CA). Genetic analyses of HEV sequences were conducted using the neighbor-joining method with GeneTux-Mac Version 13 (Genetyx Corporation, Tokyo, Japan).

RESULTS

All 200 patients in this cohort were negative for IgM antibodies to HAV, HBV, and HCV but all expressed IgM anti-HEV, which indicated that all were suffering from acute hepatitis E. Table II shows the age and gender distribution, and the occupations and residences of the patients. The patients had used drinking water from multiple sources (supply water, pond water, tube well water, and water from rivers). Approximately 90% of the patients visited local, unqualified doctors (without medical graduation) when they first noticed jaundice or symptoms of acute hepatitis. After there were no improvements, they visited qualified physicians (medical graduates) in Rajshahi.

Patient symptoms were recorded on a questionnaire by the attending physicians and nurses during the patient's first visit. All patients had visible jaundice. Anorexia, nausea, and vomiting were reported by most of the patients. The clinical features of the patients are summarized in Table III [A].

Biochemical analyses showed that the levels of bilirubin were above the upper normal limit (normal limit = 0.2–0.8 mg/dl) in all patients (Table III [B]). The levels of ALT varied from 41 to 2,971 IU/L (723 ± 532 IU/L, $N = 200$). Abdominal ultrasonography detected no features of liver cirrhosis or hepatocellular carcinoma in the patients. One patient died of acute liver failure 3 days after presentation. This patient was a 21-year-old woman who had given birth to her first child 2 months earlier. Twenty patients expressed HBsAg and 14 were positive for IgG anti-HCV.

Patients With Acute Hepatitis E Were Infected With HEV Genotype 1a

In 15 patients, the HEV genotype was determined by analyzing the nt sequences of the HEV genome. An image representing HEV genome is shown in Figure 1. The sequence analysis based on the PCR-amplified products indicated that all 15 HEV isolates belonged to HEV genotype 1a. A phylogenetic tree of HEV was constructed based on the 266-nt sequences of ORF 2 from the 15 HEV isolates (E01-Ban 10 to E15-Ban10, DDBL/EMBL/GenBank accession numbers AB720034–AB720048) collected in this study (Fig. 2). These 15 patients formed a single cluster in the phylogenetic tree (indicated by the dotted square in Fig. 2). Romanò et al. [2011] reported that the HEV genotypes of Italians who developed acute hepatitis E after travelling to Bangladesh. The HEV sequences of 15 HEV isolates from Rajshahi, Bangladesh shared close homology with the HEV isolates of Italian travelers infected with HEV in

TABLE I. Hepatitis E Virus-Specific Oligonucleotide Primers Used in this Study

| Primers | Stage-polarity | Nucleotide sequence (5'–3') |
|---------------|----------------|-----------------------------|
| Primer set 1 | 1st sense | GCAGACCACRTATGTGKTCG |
| | 2nd sense | GCAGACCACRTATGTGKTCG |
| | 1st antisense | TGGAGGGCAGCRTAAAGCCG |
| Primer set 2 | 2nd antisense | GTCATRCCATGGCGGAACAT |
| | 1st sense | CCTAATGTGGTCCACCGCTG |
| | 2nd sense | GACCTCGGTRGACCGGGGGTA |
| Primer set 3 | 1st antisense | GTTCCAGCGYTTGGTATACTGC |
| | 2nd antisense | TGGCTCCGGGGCKGCCGTGAG |
| | 1st sense | GCAGGCGYTTGTGGTGACG |
| Primer set 4 | 2nd sense | CATCCCCTTRGATATAGCCTG |
| | 1st antisense | GAGGGTGAYACYAGTGCTGG |
| | 2nd antisense | GCGGCAGTRATGACAGCTGT |
| Primer set 5 | 1st sense | ACCTCATGCTCCACTAAGTC |
| | 2nd sense | TCCTGRCCMAGCCACTTCAT |
| | 1st antisense | GTCCCTGYCCATATYTGGA |
| Primer set 6 | 2nd antisense | CARTGGCAGGGGCCGACTC |
| | 1st sense | CTTYGAGAAGTCCGGCCGTGA |
| | 2nd sense | GTCTCRCARTCGATCCGCCC |
| Primer set 7 | 1st antisense | GCYCATGTGYAGGCGCTGGCT |
| | 2nd antisense | TTTACTGTGGCGGTGAGCCG |
| | 1st sense | GCCTATGAGGGGTCYGATGT |
| Primer set 8 | 2nd sense | TTAGCCGACTCCCARACATG |
| | 1st antisense | AGTGACATMTCTGGTCCCTA |
| | 2nd antisense | AGYCCCTCAGGRTGGAACCA |
| Primer set 9 | 1st sense | AGTCAGAGCACTATGGCCGC |
| | 2nd sense | CRACTRAACAGCGAGCCGGC |
| | 1st antisense | CGCTATGTGCTGCCGGGCT |
| Primer set 10 | 2nd antisense | CCRYCTGGTGGGTATGGCC |
| | 1st sense | GAGAGCACACTYTACACCCG |
| | 2nd sense | TATATGCCGGTCCCGAGGAG |
| Primer set 11 | 1st antisense | ACTTGGTCCGARGTTGATGC |
| | 2nd antisense | CTTTGGGTTATGTTCCAACCTATA |
| | 1st sense | TAATGTTGACCAYCGCCCTGG |
| Primer set 12 | 2nd sense | GGCACACCTGCRGTAACCTG |
| | 1st antisense | TGCTGCCTCTTTGTGATGC |
| | 2nd antisense | CCCGCCGACRTCTGTGGC |
| Primer set 13 | 1st sense | TTTGACGCCTGGGAGCGGAA |
| | 2nd sense | TGATGGCGGGRACGAGCCC |
| | 1st antisense | CARATGGTTYGAGGCCAATA |
| Primer set 14 | 2nd antisense | ACGGTGGCGGCGCTGCAT |
| | 1st sense | GGACGTTGTGTTGGTYCCGAC |
| | 2nd sense | CCTGGTGCCTCAATGATGAC |
| Primer set 15 | 1st antisense | TGCGTAAYGCCTGGCGCCG |
| | 2nd antisense | ACTTCTCAGTGTGGCGCGTC |
| | 1st sense | GATYCAGACCACTAGYCGGGT |
| Primer set 16 | 2nd sense | CRACACTATCACAGGYGGTG |
| | 1st antisense | GAAAYTAGTGTTCACCCAGGC |
| | 2nd antisense | AGGTAGAGRAGGCCCTGTTT |
| Primer set 17 | 1st sense | AATGTYGACACCYTGGCTGCC |
| | 2nd sense | TCGAGCTCAAGGACGGCGGAG |
| | 1st antisense | CCNCCGTCTTGCCAGATTAG |
| Primer set 18 | 2nd antisense | TGGCCCTTCTCRACCATGGC |
| | 1st sense | CGAGNCAGCGCAAGGCCGT |
| | 2nd sense | TCCWCCATAATAGCACACTC |
| Primer set 19 | 1st antisense | GTYCGCGACTCTCTCGCCCG |
| | 2nd antisense | CAGARAARTCATTCTCAAACACCAT |
| | 1st sense | CATGGYAAAGTGGGYCAGGG |
| Primer set 20 | 2nd sense | ACGRTACTACTGCAAAGCAC |
| | 1st antisense | TTCCGYGCTATTGAGAAGGC |
| | 2nd antisense | CGAATCATCACCTTTAAARGC |
| Primer set 21 | 1st sense | GATYYTGCAGGCCCGGAAGGA |
| | 2nd sense | ATRCCAATCAGGTTATGAAC |
| | 1st antisense | CACTCCGGTGAGCCYGGCAC |
| Primer set 22 | 2nd antisense | GGGGARACCCCATARACACG |
| | 1st sense | GGTGTGTGGTGGCCCCCGG |
| | 2nd sense | GGCGAAGGGGTTGGTTGGATG |
| Primer set 23 | 1st antisense | ACYGAGAAGAATTGGGGCCC |
| | 2nd antisense | TAGGGGATTGCGAAGGGCTGAG |

(continued)