known contact with foreigners or travelers who had been abroad, no contact with pigs and other animals, and no history of blood transfusion. Patient 7 had consumed meat/viscera from a wild boar that he had captured himself as a hunter approximately 2 months before disease onset.[24] Patient 11 ingested liver from a wild boar in Aichi, the neighboring prefecture to Mie, where the patient lived. Of note, one patient (no. 4) ingested pig liver 1 month before developing hepatitis E, and two other patients (nos. 1 and 2) reported eating liver or intestine from pigs 4–5 weeks before the onset of disease, although it could not be ruled out that the liver and intestine were derived from cows. Patient 16 ate barbecued pork. Although the risk factors were unknown for eight patients, seven patients (nos. 5, 6, 8 and 12–15) reported consumption of raw fish (sashimi and/or sushi) with or without raw shellfish 1–2 months before the onset of disease, as most Japanese people have a habit of eating raw fish and/or shellfish.

Detection of HEV RNA in raw pig liver intended for human consumption

To investigate whether raw pig liver used as food in Mie where the patients lived is contaminated with HEV and to examine whether the HEV strains recovered from the patients are phylogenetically associated with those circulating in pigs, which are considered to be the major animal reservoirs for HEV in Japan, a total of 243 packages of raw pig liver that were purchased from grocery stores in three cities (Yokkaichi, Suzuka and Tsu) in Mie where our patients lived (Fig. 1), were tested for the presence of HEV RNA (Table 3). Pig liver specimens from 12 (4.9%) of the 243 packages had detectable HEV RNA, with the positivity for HEV RNA being detected in nine (56%) of 16 stores and in all three cities tested. The HEV load was estimated to be 2.9×10^6 copies/g for pig liver sample no. 012 and 3.9×10^4 copies/g for pig liver sample no. 047, while those of the remaining 10 HEV RNA positive pig liver specimens having low virus loads of less than 4.0×10^3 copies/g. The amplification products of ORF2 (412 nt; primer sequences at both ends excluded) from the 12 HEV RNA positive pig liver specimens were sequenced and compared (Table 3). All 12 swine HEV isolates segregated into genotype 3, differing by 0–14.1% from each other within the 412-nt ORF2 sequence.

Although pig liver sample nos. 021 and 029 were purchased from the same store (Store P) on different days (1 or 15 September 2011), the swJLMie021 and swJLMie029 isolates had identical sequences, suggesting that slices of pig liver in the no. 021 and 029 packages were derived from pigs from the same farm. Because pig

Table 3 Detection of HEV RNA and the HEV genotypes in pig liver sold as food in 16 grocery stores in Mie, Japan

City	Store†	No. of liver samples tested	HEV RNA positive sample(s)	HEV subgenotype (isolate name)‡
Yokkaichi	A	10	1 (10.0%)	3a (204)
	B	10	1 (10.0%)	3a (205)
	C	9	2 (22.2%)	3a (220), 3a (228)
	D	5	0	
	E	3	0	
	F	2	0	
	G	7	0	
	H	4	0	
Suzuka	I	34	0	
	J	33	1 (3.0%)	3b (193)
	K	31	1 (3.2%)	3a (047)
	L	24	0	
	M	21	1 (4.8%)	3b (012)
	N	17	2 (11.8%)	3b (069), 3b (162)
Tsu	O	21	1 (4.8%)	3b (152)
	P	12	2 (16.7%)	3b (021), 3b (029)
Total	16	243	12 (4.9%)	

†Pig liver packages in stores A–F were from Aichi, while those in stores G–P were local products in Mie.
‡For simplicity, the prefix "swJLMie" is excluded.
HEV, hepatitis E virus.

liver sample nos. 220 and 228 were also purchased from the same store (Store C) on different days (23 December 2012 or 26 January 2013) and had HEV strains (swJLMie220 and swJLMie228 isolates, respectively) that were 99.8% identical to each other, it is likely that the slices of pig liver in the no. 220 and 228 packages were also derived from pigs from the same farm. The swJLMie204 and swJLMie205 isolates had the same 412-nt sequence, but they were isolated from slices or a block of pig liver purchased from different stores (Store A or B) on the same day (23 September 2012), suggesting that pig liver package nos. 204 and 205 were derived from the livers of distinct pigs, but from the same swine herd. Although pig liver sample nos. 152 and 193 were purchased on different days (28 April 2012 and 24 July 2012) in different stores (Store J or O), the swJLMie152 and swJLMie193 shared 99.5% identity, probably due to the circulation of the same swine HEV strain on multiple farms or the sale of pig livers from a single farm in multiple stores.

Genetic analysis of HEV strains recovered from raw pig liver intended for food, and comparison with those from hepatitis patients in Mie

The 12 swine HEV isolates obtained in the present study were exclusively grouped into genotype 3. Five isolates were further segregated into subgenotype 3a, and the remaining seven isolates segregated into subgenotype 3b (Table 3). When these 12 swine HEV isolates were compared with the human HEV isolates of Japanese or non-Japanese origin, including those obtained in the present study, two 3b swine HEV isolates (swJLMie152 and swJLMie193) obtained from pig liver package nos. 152 and 193, had nucleotide sequence identity of 99.5–100% with the HE-JA12-0483 and HE-JA12-0940 isolates recovered from patients 13 and 17, respectively (see Tables 1,2). The remaining five 3b swine HEV isolates were closest to reported Japan-indigenous HEV isolates, with the highest nucleotide sequence similarity ranging 93.4–96.1%, but these were only 87.3–92.4% identical to HE-JA05-0753 and HE-JA11-0975 recovered from patients 2 and 10, respectively, in the present study. Although 3a swine HEV strains were obtained from five liver specimens, no. 3a HEV strains were recovered from hepatitis E patients in the present study (Table 4). These five 3a swine HEV isolates were closest to reported HEV isolates of Japanese origin, with the highest nucleotide sequence identity being 92.0–97.3%.

A phylogenetic tree was constructed based on the common 412-nt ORF2 sequence of representative human and animal HEV isolates of Japanese or non-Japanese origin, including those obtained in the present study, and the 12 swine HEV isolates obtained in the present study (Fig. 2). As illustrated in Figure 2, swJLMie152 and swJLMie193 were most closely related to HE-JA12-0483 and HE-JA12-0940, and formed a cluster supported by a high bootstrap value of 99%. Of note, the predominant HEV strains of subgenotype 3e recovered from 10 hepatitis E patients segregated into a cluster supported by a bootstrap value of 99%. The HE-JA07-0229 isolate obtained from patient 3 segregated into a cluster within genotype 4, consisting of Chinese human and swine HEV isolates, with a high bootstrap value of 94% (Fig. 3). This finding indicates the Chinese origin of the HE-JA07-0229 isolate and the importation of this isolate through travel to China by patient 3. The observed phylogenetic relationship between the 17 human HEV strains obtained from hepatitis E patients in Mie and the 12 swine HEV strains obtained from liver specimens in the present study was

Table 4 Genotype/subgenotype distribution of HEV strains obtained from pig liver sold as food and from patients with acute hepatitis E in Mie, Japan

City	Genotype/subgenotype of HEV strains from pig liver sold as food			Genotype/subgenotype of HEV strains recovered from acute hepatitis E patients			
	3a	3b	3e	3a	3b	3e	4
Kuwana	0	0	0	0	1	0	0
Yokkaichi	4	0	0	0	1	2	0
Suzuka	1	4	0	0	2	7	2
Tsu	0	3	0	0	0	2	0
Total	5	7	0	0	4	11	2

HEV, hepatitis E virus.

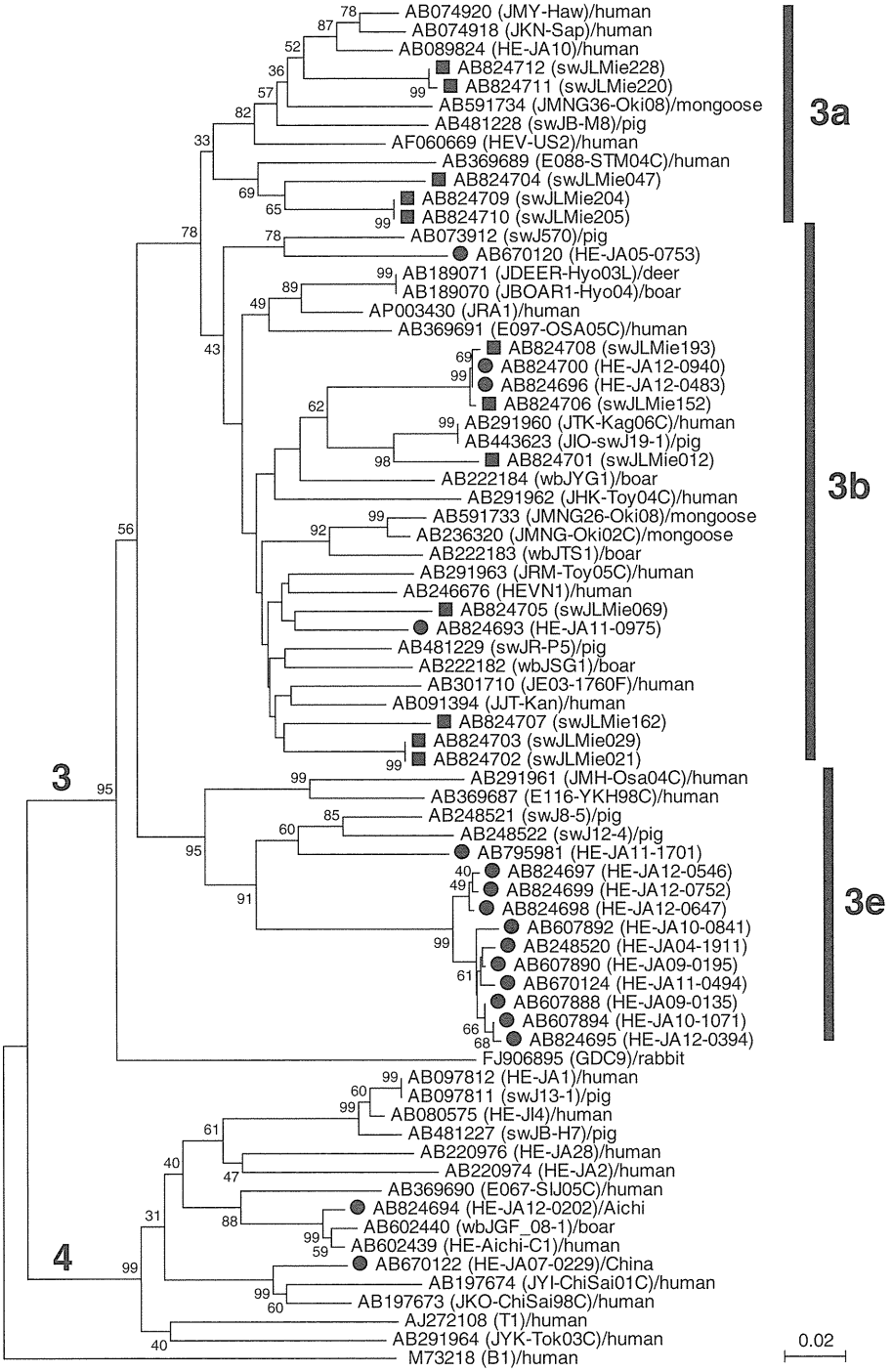

Figure 2 Neighbor-joining tree of the 412-nt open reading frame (ORF)2 sequences of the 17 human and 12 swine hepatitis E virus (HEV) strains obtained in this study, with 42 reference sequences of genotypes 3 and 4 and an outgroup isolate of genotype 1 (M73218). The reference sequences are shown with accession nos., followed by the isolate name, and the name of the animal species from which it was isolated. Japan-indigenous genotype 3 isolates are divided into three subgenotypes: 3a, 3b and 3e.[9] The 17 human and 12 swine HEV strains isolated in this study are marked with closed circles and closed boxes, respectively. The bootstrap values are indicated for the nodes as a percentage of the data obtained from 1000 resamplings. The scale bar is in units of nucleotide substitutions per site.

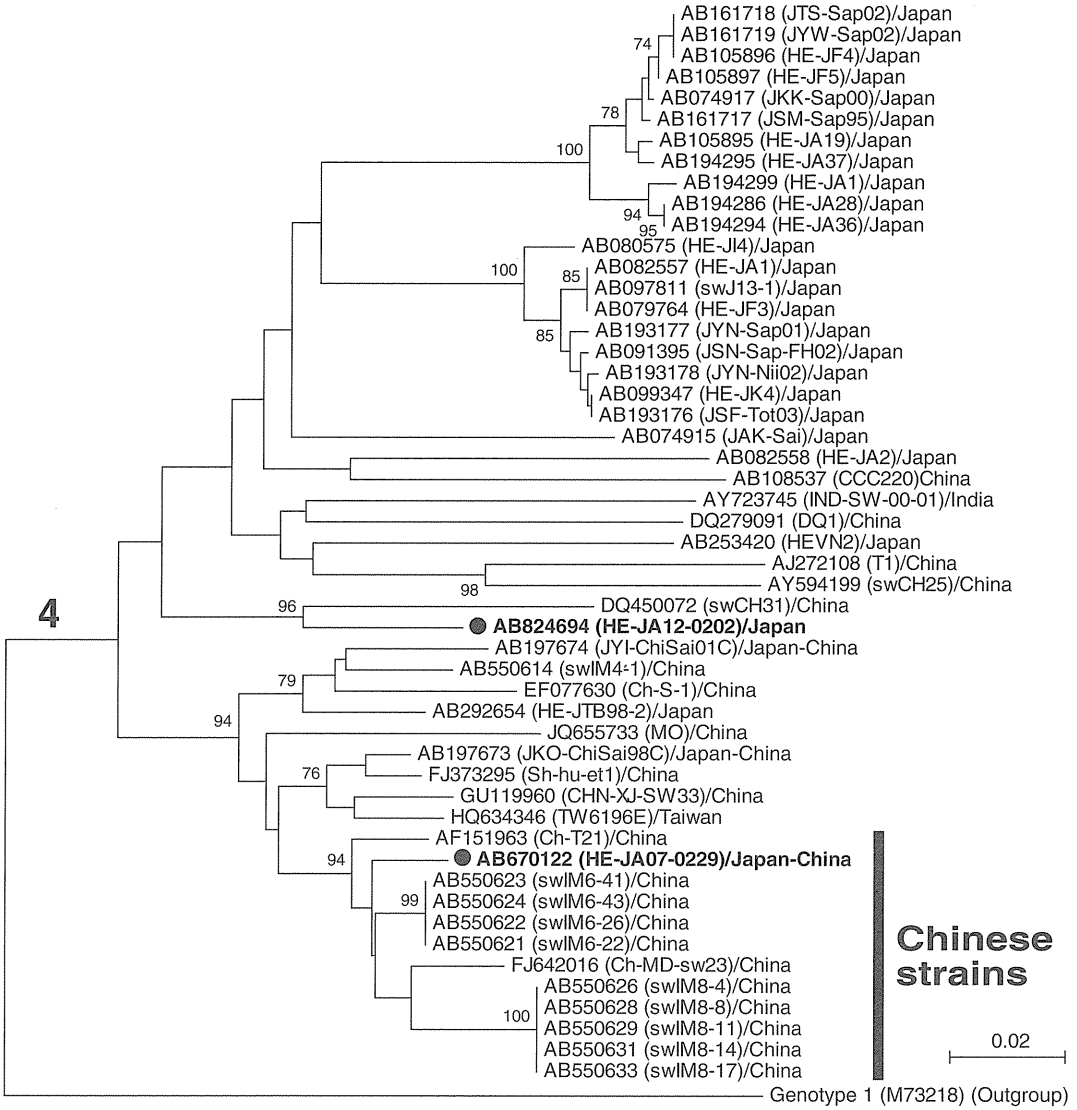

Figure 3 Neighbor-joining tree of the 412-nt reading frame (ORF)2 sequences of the genotype 4 hepatitis E virus (HEV) strains (HE-JA07-0229 and HE-JA12-0202) obtained in this study, with 49 reference sequences of genotype 4 and an outgroup isolate of genotype 1 (M73218). The reference sequences are shown with accession nos., followed by the isolate name in parentheses, and the name of countries from which it was isolated. The two genotype 4 HEV strains isolated in this study are marked with closed circles. The bootstrap values of >70% are indicated for the nodes as a percentage of the data obtained from 1000 resamplings. The scale bar is in units of nucleotide substitutions per site.

confirmed by another phylogenetic tree constructed based on the ORF1 412-nt sequence (Fig. 4).

DISCUSSION

IN THE PRESENT study, polyphyletic HEV strains were isolated from patients with sporadic acute hepatitis E between 2004 and 2012 in Mie prefecture (Fig. 1), including European-type subgenotype 3e HEV strains, which accounted for 65% (11/17) of the total strains isolated, followed by subgenotype 3b strains ($n = 4$) and genotype 4 strains ($n = 2$). These results confirmed our previous studies with small numbers of patients reporting the predominance of rare subgenotype 3e strains in Mie.[16,17] Furthermore, the present study revealed that raw pig liver sold in local grocery stores in Mie was

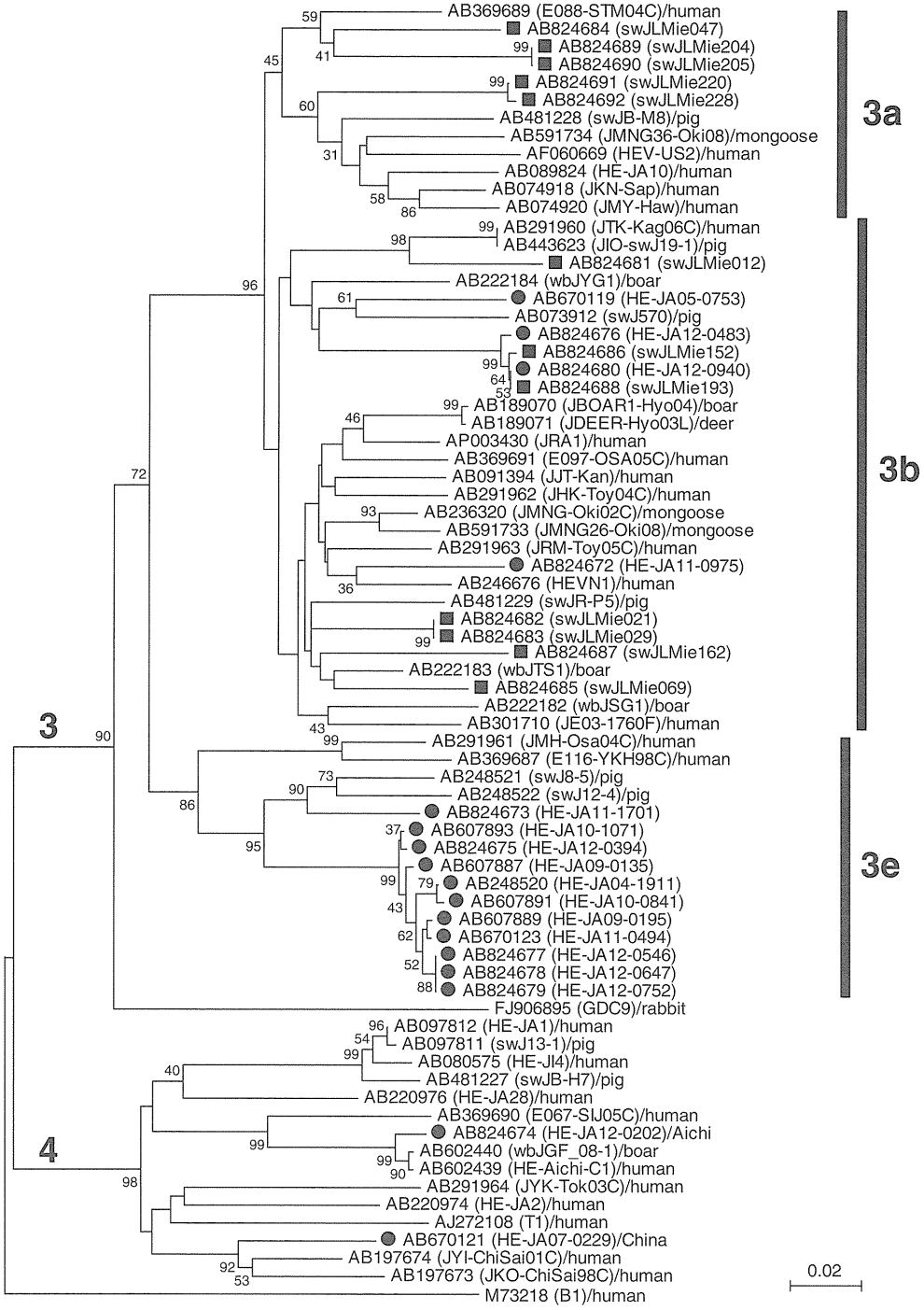

Figure 4 Neighbor-joining tree of the 412-nt open reading frame (ORF)1 sequences of the 17 human and 12 swine hepatitis E virus (HEV) strains obtained in this study, with 42 reference sequences of genotypes 3 and 4 and an outgroup isolate of genotype 1 (M73218). The reference sequences are shown with accession nos., followed by the isolate name, and the name of the animal species from which it was isolated. Japan-indigenous genotype 3 isolates are divided into three subgenotypes: 3a, 3b and 3e.[9] The 17 human and 12 swine HEV strains isolated in this study are marked with closed circles and closed boxes, respectively. The bootstrap values are indicated for the nodes as a percentage of the data obtained from 1000 resamplings. The scale bar is in units of nucleotide substitutions per site.

contaminated with HEV at a frequency of 4.9% (12/243). Although 3e HEV strains were not identified from the purchased pig liver packages in the present study, two swine strains from the pig liver specimens and two human strains from the hepatitis E patients in Mie, belonging to subgenotype 3b, were found to be closely related to each other, with nucleotide sequence identities of 99.5–100%, suggesting the importance of pigs as reservoirs for HEV infection in humans, including the recent cases in Mie.

Nationwide surveys revealed that genotype 3 is the most prevalent HEV genotype infecting humans, swine and wild boars in Japan.[14] Japan-indigenous genotype 3 HEV strains are divided into two major subgenotypes (3a and 3b); one minor subgenotype (3e); and a few other unassigned lineages.[7–9,17] The Japan-indigenous subgenotype 3e strains are closely related to European strains, and are usually comparatively rare in the humans, swine and wild boars in Japan.[25,26] The Mie HEV strains recovered from hepatitis E patients in the present study were found to be unique, in that more than half the HEV strains (65% or 11/17) belonged to subgenotype 3e, further classifiable into two lineages within subgenotype 3e (Figs 2,4). These consisted of the HE-JA11-1701 isolate and the remaining 10 isolates, respectively. The major 3e lineage is represented by the HE-JA04-1911 isolate, which was isolated in 2004, and whose entire genomic sequence has been determined.[25]

Based on the phylogenetic structure and the results of the coalescent analyses, it has been suggested that the subgenotype 3e isolates entered Japan from Europe by importation of large-race pigs around 1966, and that several lineages of subgenotype 3e expanded to wide areas of Japan around 1992, and one of the lineages was indigenized in wild boars in Mie prefecture between 1992 and 2009.[26] As reported previously, the HE-JA11-1701 isolate representing the minor 3e lineage was recovered from a hunter who developed sporadic acute hepatitis E approximately 2 months after consumption of meat/viscera from a wild boar, and this was highly similar to a HEV isolate (JBOAR012-Mie08) that had been isolated from a wild boar captured near the patient's hunting area, thereby strongly suggesting that the source of HEV infection in this patient was an HEV-infected wild boar.[24] Of note, the remaining 10 subgenotype 3e strains obtained during the past 8 years between July 2004 and July 2012 in the present study were 97.6–99.8% identical to each other, suggesting the indigenousness and maintenance of the 3e HEV strains circulating in Mie. However, these 3e human strains were not homologous to those obtained from wild boars in Mie, and formed a cluster separate from that of wild boars.[26] Because several lineages of genotype 3 HEV strains have been isolated from wild boars in the same area,[27] and meats from wild boars are commercially available in grocery stores in some rural areas in Mie, near the hunting areas, further efforts are warranted to identify the 3e strains from wild boars in Mie that are homologous to those from hepatitis patients, if such strains exist.

Two hepatitis patients (nos. 3 and 11: Table 2) in the present study contracted infections of genotype 4 HEV. One patient (no. 3) was presumed to have been infected with HEV while traveling in China where he consumed raw vegetables and sushi (raw fish and shellfish). In support of our speculation, the genotype 4 HEV obtained from this patient formed a cluster with Chinese human and swine genotype 4 strains, which was supported by a high bootstrap value in the phylogenetic tree constructed based on the ORF2 sequence (Fig. 3). Another patient (no. 11) infected with genotype 4 HEV had a history of ingesting liver from wild boar in Aichi prefecture, where four patients had been reported to have developed acute hepatitis E after consumption of wild boar meat[28] and homologous genotype 4 HEV strains have been obtained from wild boars in the neighboring prefecture.[29,30] High genomic similarity between genotype 4 HEV strains isolated from our patient and those previously reported from Aichi may support the zoonotic food-borne transmission of HEV from wild boar infected with genotype 4 HEV to our patient.

In the present study, raw pig liver as food sold in grocery stores in Mie was found to be contaminated with HEV at the frequency of 4.9% of the total examined packages (12/243). The detection of HEV RNA in raw pig liver intended for human consumption in Mie is not surprising, because contamination of commercially sold pig livers with HEV has been reported not only in Japan,[11] but also in the USA,[15] the Netherlands,[31] India,[32] France[33] and Germany.[34] However, this finding was contrary to our assumptions, because HEV RNA was detected significantly more frequently in commercially sold pig livers in Mie than in Hokkaido (4.9% vs 1.9% [7/363], $P = 0.0372$), where hepatitis E is endemic and approximately one-third of hepatitis E patients in Japan have been reported annually.[14] Some Japanese people have a habit of eating raw pig liver, and it is served at some restaurants in Japan. Based on the evidence that HEV infection is distributed widely in domestic pigs in Japan,[8,35] it is very likely that the raw pig livers as food sold in grocery stores or supermarkets throughout Japan

are contaminated with HEV, although the rate of virus contamination may differ by region, and should be examined in various areas in Japan, including both endemic and non-endemic regions (northern and southern parts, respectively, of Japan),[36] to assess the actual risk of HEV transmission from pig livers to humans. Importantly, the contaminating virus in commercial pig livers sold in local grocery stores remains infectious when inoculated into pigs[15] and cultured cells.[37]

Of note, the virus sequences recovered from pig livers (nos. 152 and 193) were 99.5–100% identical to the viruses recovered from hepatitis E patients (nos. 13 and 17). However, these two patients did not remember consuming pig liver before the onset of hepatitis E (Table 2). The route of HEV transmission was unknown for patient nos. 13 and 17, although patient no. 17 reported frequent ingestion of raw horse meat and sushi. The HEV sequences recovered from the two patients and two pig liver specimens differed by 7.8% or more from the deposited HEV sequences as of June 2013, thus suggesting the uniqueness of these human and swine HEV sequences, and that the source of the HEV in the patients was likely pigs. It is now evident that pigs constitute a major reservoir, and are able to shed the virus into the environment.[12,38]

Contrary to our expectation, the distribution of HEV genotype/subgenotype was different between hepatitis E patients and purchased pig liver packages (Table 4). The reason for this discrepancy remains unknown. However, it is likely that eating habits and source of infection affect the prevalence of clinical and subclinical HEV infection and the distribution of HEV genotype/subgenotype. In support of our speculation, in the present study in Mie, only one patient (no. 4) reported consumption of cooked pig liver before the disease onset, and two additional patients (nos. 1 and 2) ingested raw liver or cooked intestine from animals, although it was unclear whether the viscera originated from pigs or cows. On the other hand, in Hokkaido where hepatitis E is endemic, approximately 70% of hepatitis E patients have a history of eating uncooked or undercooked liver and/or colon/intestine from pigs,[14] and the HEV sequences recovered from commercial pig liver are closely related to, or identical in a few cases, to the viruses recovered from hepatitis E patients who ingested pig liver/intestine before the onset of the disease.[14]

Hepatitis E virus replicates in the liver and gastrointestinal tract,[39,40] and thus infected animals such as pigs excrete large amounts of HEV in feces, which poses a concern for environmental safety. Sewage water from a pig slaughterhouse in Spain was shown to contain genotype 3 HEV that was similar to the indigenous Spanish human strain, and HEV has been repeatedly detected in pig manure storage facilities.[41] In the USA, concrete pits and lagoons that served as storage facilities were found to be positive for genotype 3 HEV, which could subsequently contaminate water and even spread across the species barrier.[42] Of interest, in South Korea, oysters have been shown to be contaminated with genotype 3 HEV that is homologous to the HEV from the Korean pigs.[43] Ishida *et al.*[44] reported that genotype 3 HEV was detected in a sewage sample and a seawater sample in Japan. In other reports, the isolation of HEV from sewage and river water raised the possibility of the contamination of shellfish by infectious HEV.[45,46] Therefore, river water contaminated with swine feces or incompletely sanitized sewage may prove to be the principal source of HEV contamination in shellfish. The HEV that is abundant in the pig population can be shed into the environment, and, directly or indirectly, be transmitted to humans. Further studies are needed to elucidate the source of HEV infection in hepatitis E patients in Mie by analyzing the presence of the virus in pig populations and environmental reservoirs that are homologous to those in patients.

In conclusion, the predominant HEV strains in hepatitis E patients in Mie belonged to subgenotype 3e, that is rare in Japan. HEV RNA was detected in approximately 5% of the pig liver sold as food in Mie. The HEV sequences recovered from two pig liver specimens were 99.5–100% identical to the viruses recovered from two patients who developed sporadic acute hepatitis E independently, indicating that pigs play an important role as animal reservoirs for HEV infection in humans in Mie. However, these two patients did not recall consuming pig liver before the onset of hepatitis E, and the route of HEV transmission was unknown for these patients, and no subgenotype 3e HEV strains that were prevalent in hepatitis patients in Mie were identified from the pig liver specimens studied. To prevent a future endemic or epidemic of HEV infection, further detection and characterization of HEV strains in animal and environmental reservoirs are warranted in Mie, as well as in other prefectures of Japan, where domestic hepatitis E has been increasingly reported.[14]

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Hepatology Research

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Case Report

High genomic similarity between European type hepatitis E virus subgenotype 3e strains isolated from an acute hepatitis patient and a wild boar in Mie, Japan

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A 67-year-old male living in Tsu city, Mie prefecture, Japan was referred to our hospital for further examination of acute liver injury and was diagnosed as having clinical hepatitis E virus (HEV) infection in January 2010. The HEV strain (HE-JA11-1701) isolated from the patient belonged to genotype 3 and European-type subgenotype 3e. It was presumed that the patient had been infected from a wild boar (*Sus scrofa leucomystax*) because he consumed meat/viscera from a wild boar that he had captured himself as a hunter approximately 2 months before disease onset. A specimen of the boar meat/viscera that the patient had ingested was not available. However, the HE-JA11-1701 strain was 99.8% identical within the 412-nucleotide sequence of the open reading frame 2 region to a HEV strain (JBOAR012-Mie08) that had been recovered from a wild boar captured near the patient's hunting area in 2008. A phylogenetic analysis confirmed that the two HEV strains had a close genetic relationship and were segregated into subgenotype 3e, supported by a high bootstrap value of 99%. Of note, the HE-JA11-1701 and JBOAR012-Mie08 strains were remotely related to the 3e strains reported in Japan and European countries, with a nucleotide difference of 7.9–13.9%, reinforcing the uniqueness of the 3e strains obtained in the present study. These results strongly support our speculation that the patient developed acute hepatitis E via consumption of HEV-infected boar meat/viscera. Genetic analyses of HEV strains are useful for tracing infectious sources in sporadic cases of acute hepatitis E.

Key words: hepatitis E virus, Japan, nucleotide sequence, subgenotype 3e, wild boar

INTRODUCTION

HEPATITIS E VIRUS (HEV) is transmitted via the fecal–oral route through the consumption of contaminated water or food. HEV infection had long been considered to be restricted to developing countries where sanitation conditions are suboptimal. However, HEV is now recognized to be an important pathogen of acute hepatitis in industrialized countries. Both HEV and hepatitis caused by HEV appear to exist virtually everywhere worldwide.[1] In Japan, autochthonous HEV strains were first recovered in 2001 from a Japanese patient with sporadic acute hepatitis E who had no history of traveling abroad and from domesticated pigs independently.[2,3] Zoonotic food-borne transmission of HEV via the ingestion of meat or viscera of infected animals including pigs, wild boar and deer is the main route of HEV transmission in Japan,[4–6] where transfusion-transmitted HEV infection is reported to rarely occur.[7,8] However, infectious source(s)/route(s) remain unknown in nearly half of hepatitis E cases in Japan.[4]

Four major genotypes of HEV that infect humans have been identified thus far.[9] Unlike genotypes 1 and 2 that are responsible for the majority of HEV infections in

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developing countries, both genotypes 3 and 4 cause acute sporadic hepatitis in industrialized countries and some developing countries and exhibit the characteristics of zoonosis. Pigs are the most frequent HEV reservoir among animals in Japan.[10,11] HEV has been isolated from the serum and liver specimens of wild boars,[12,13] and many cases of hepatitis E that developed after the patients ate wild boar meat have been reported,[14–21] with direct evidence of HEV infection occurring via the consumption of boar meat.[14] Hence, it is beyond doubt that wild boars serve as another important reservoir for HEV in humans.

Autochthonous HEV strains obtained from humans and animals in Japan belong to genotype 3 or 4, and Japan-indigenous genotype 3 HEV strains have been provisionally classified into three subgenotypes: 3b (3jp), 3a (3us) and 3e (3sp), where "jp" stands for Japan-type, "us" for US-type and "sp" for Spanish (European) type.[6,22,23]

Recently, we experienced a male patient from Mie prefecture, Japan, with sporadic acute hepatitis E (Fig. 1). The patient used to hunt wild boars as a hobby and would consume the meat/viscera of the captured boars. Because there were no leftovers of the boar meat and viscera that the patient had ingested before the onset of disease, the nucleotide (nt) sequence of the HEV strain recovered from the patient was compared with that of a HEV strain obtained from an infected wild boar that had been captured near the patient's hunting area in Mie prefecture. Based on a high genomic similarity between the HEV strains of the rare subgenotype 3e obtained from the patient and the wild boar, we herein report the case of a hunter who developed sporadic acute hepatitis E, most likely via the consumption of meat/viscera from a captured boar.

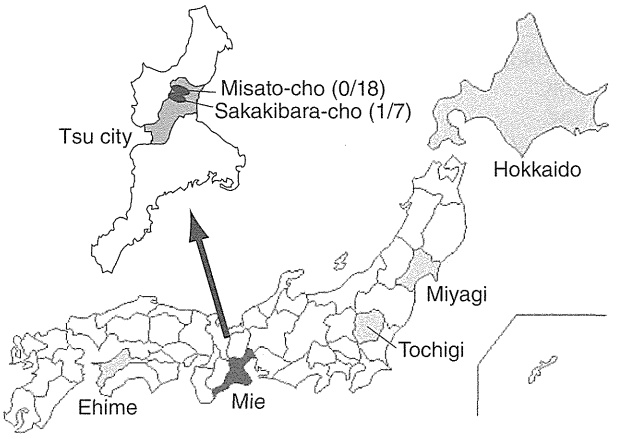

Figure 1 Map of Japan showing five prefectures where the 3e hepatitis E virus (HEV) strains have been identified and a magnified map of Mie prefecture indicating Tsu city with two hunting areas (Misato-cho and Sakakibara-cho). A total of 25 wild boars were captured during 2008–2009, and their serum samples were subjected to HEV RNA detection. Misato-cho was the patient's hunting area and Sakakibara-cho was the area where the wild boar (JBOAR012-Mie08) was captured. Between these two neighboring regions, there are no obstacles, such as large rivers, mountains or roads, that would prevent wild boars from movement. The numbers before and after the slashes in parenthesis indicate the numbers of samples positive for HEV RNA and those that were tested for the presence of HEV RNA in the present study, respectively.

CASE REPORT

IN JANUARY 2010, a 67-year-old man was referred to our hospital with a clinical diagnosis of acute liver injury. He had no symptoms at the referral visit; however, his laboratory data revealed an elevation of the serum liver enzyme levels (aspartate aminotransferase, 616 IU/L; alanine aminotransferase, 1218 IU/L; lactate dehydrogenase, 569 IU/L; alkaline phosphatase, 354 IU/L; and γ-glutamyltransferase, 94 IU/L). The prothrombin time was within the normal range (100%) and the total bilirubin level was normal (0.6 mg/dL) (Table 1). The patient had no history of traveling abroad, receiving blood or blood-related products, or injection drug use. Of note, he used to hunt wild boars as a hobby and would slaughter the boars himself. He had ingested the meat/viscera of a wild boar captured at the Misato-cho area in Mie prefecture approximately 2 months before the onset of acute hepatitis (Fig. 1). Although the viral markers of hepatitis A, B and C were negative in the serum, the patient had the immunoglobulin (Ig)M, IgA and IgG classes of anti-HEV antibodies detectable by an in-house enzyme-linked immunoassay with recombinant open reading frame 2 (ORF2) protein,[24] as well as HEV RNA detectable by nested reverse transcription polymerase chain reaction with primers targeting the ORF2 region,[5] which led to the diagnosis of acute hepatitis E. The patient recovered within 1 month after disease onset without any sequelae.

The 412-nucleotide (nt) sequence within the ORF2 region of the HEV strain (HE-JA11-1701) recovered from the patient was identified using a previously described method,[5] and deposited to the GenBank/EMBL/DDBJ databases under accession no. AB795981. The HE-JA11-1701 strain belonged to genotype 3 and was most similar to European type subgenotype 3e HEV strains, with an identity of 86.4%, 91.0% and

Table 1 Laboratory data at presentation to our hospital

Hematology		Electrolytes and renal function	
WBC	$3960 \times 10^3/\mu L$	Na	140 mEq/L
RBC	$485 \times 10^6/\mu L$	K	4.4 mEq/L
Hemoglobin	14.7 g/dL	Cl	100 mEq/L
Hematocrit	45.5%	BUN	13 mg/dL
Platelet	$220 \times 10^3/\mu L$	Creatinine	0.82 mg/dL
Neutrophil	63%	Uric acid	6.0 mg/dL
Lymphocyte	27%		
Monocyte	5%	Blood coagulation	
Eosinophil	4%	Prothrombin time	100%
Basophil	1%		
Blood chemistry		Virus markers	
T-Bil	0.6 mg/dL	IgM-HAV Ab	(−)
D-Bil	0.2 mg/dL	HBsAg	(−)
AST	616 IU/L	HCV Ab	(−)
ALT	1218 IU/L	IgG anti-HEV Ab	0.513 (+)
LDH	569 IU/L	IgM anti-HEV Ab	1.568 (+)
γ-GT	94 IU/L	IgA anti-HEV Ab	1.833 (+)
ALP	354 U/L	HEV RNA	(+)
LAP	91 IU/L		
Ch-E	278 IU/L	Autoantibodies	
Total protein	7.2 g/dL	ANA	(−)
Albumin	4.5 g/dL		
T-chol	196 mg/dL		
Triglyceride	50 mg/dL		
Blood sugar	137 mg/dL		

γ-GT, γ-glutamyltransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANA, antinuclear antibodies; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Ch-E, cholinesterase; D-Bil, direct bilirubin; HBsAg, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; IgM HAV Ab, immunoglobulin M-hepatitis A antibody; LAP, leucine aminopeptidase; LDH, lactate dehydrogenase; RBC, red blood cell count; T-Bil, total bilirubin; T-chol, total cholesterol; WBC, white blood cell count.

91.3% to the representative isolates of subgenotype 3e whose entire genomic sequences have been determined: HE-JA04-1911, swJ8-5 and swJ12-4, respectively.[25] With regard to the 26 other 3e strains reported in Japan (see Fig. 2 for the accession numbers), the HE-JA11-1701 strain shared only 86.1–91.0% identity within the overlapping 412-nt sequence. Because none of the boar meat/viscera that the patient had ingested before the onset of hepatitis remained, we attempted to detect HEV RNA in stored serum samples of 25 wild boars that had been captured around the patient's hunting area during the hunting season of autumn 2008 through spring 2009. The 25 serum samples were assessed for the presence of HEV RNA. Notably, one (JBOAR012-Mie08) of seven wild boars captured in the Sakakibara-cho area tested positive for HEV RNA, while none of the 18 wild boars captured in the Misato-cho area had detectable levels of HEV RNA (Fig. 1).

The simple homology of the 412-nt sequences between HE-JA11-1701 and JBOAR012-Mie08 (accession no. AB780455) was 99.8%, with only one nucleotide substitution between these two strains. A phylogenetic tree constructed using the neighbor-joining method[26] based on the 412-nt ORF2 sequences of the two sequences (HE-JA11-1701 and JBOAR012-Mie08) with 48 reference sequences confirmed that the HE-JA11-1701 and JBOAR012-Mie08 strains are closely related mutually and form a cluster with 34 other subgenotype 3e isolates, supported by a high bootstrap value (99%) (Fig. 2). Of note, however, the HE-JA11-1701 and JBOAR012-Mie08 strains were distantly related to reported subgenotype 3e strains recovered from six cases of sporadic acute hepatitis E in Mie prefecture.[27,28] Among these six strains, the HE-JA04-1911 strain isolated from a patient living in Tsu city shared only 86.4% identity with the HE-JA11-1701 and JBOAR012-Mie08 strains within the 412-nt ORF2 sequence.

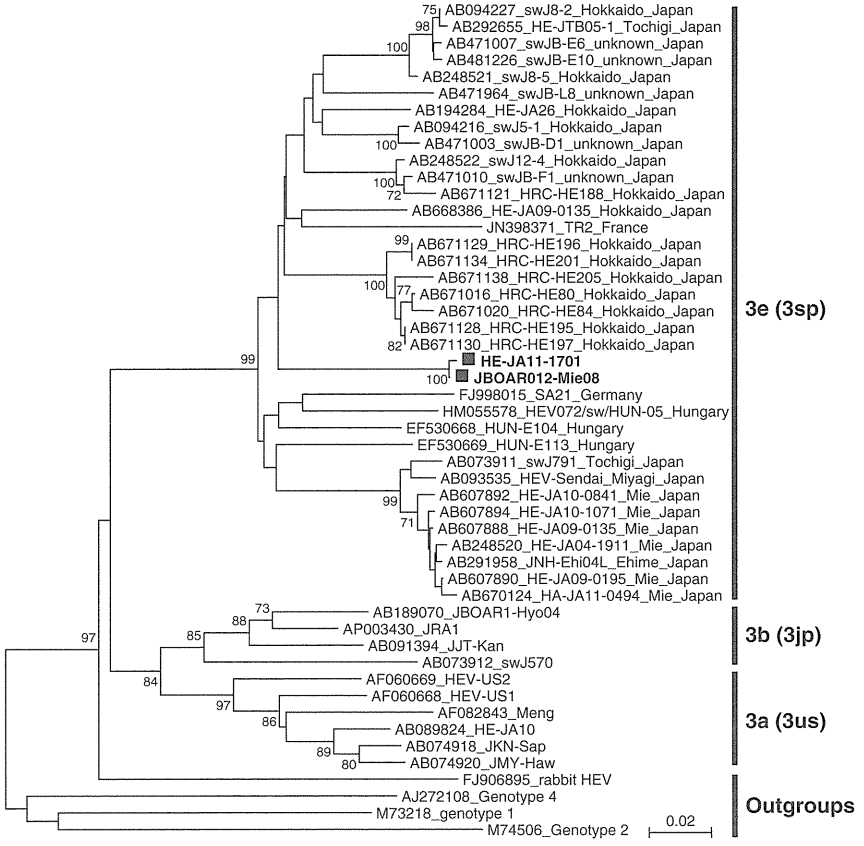

Figure 2 A neighbor-joining tree of the 412-nucleotide (nt) open reading frame 2 (ORF2) sequences of the two hepatitis E virus (HEV) isolates obtained in this study with 44 reference sequences of genotype 3 and the outgroup isolates. The reference sequences are shown with accession numbers, followed by the isolate name, and the name of the prefecture and/or country where it was isolated. Japan-indigenous genotype 3 isolates are divided into three subgenotypes: 3a (3us), 3b (3jp) and 3e (3sp).[6,23] The two 3e strains isolated in this study are shown in bold and marked with closed boxes. The bootstrap values (>70%) are indicated for the nodes as a percentage of the data obtained from 1000 resampling. The scale bar is in units of nucleotide substitutions per site.

DISCUSSION

THERE ARE AT least four HEV genotypes capable of infecting humans, and genotypes 3 and 4 are known to be autochthonous in Japan.[9] Although the presence of 10 subgenotypes (3a–3j) within genotype 3 has been proposed,[23] three subgenotypes, 3a, 3b and 3e, have been identified in hepatitis patients and domestic pigs in Japan.[6,22] Of these, 3a (3us) and 3b (3jp) are frequently observed, while 3e (3sp) is rare, in Japan.[29] In Europe, the 3e strains are known to be associated with the occurrence of acute hepatitis E as a zoonosis.[30] A coalescent analysis indicated that the import of a breed of large-race pigs from Europe since the 1960s may be responsible for the introduction of subgenotype 3e isolates to Japan.[29] The indigenization and spread of HEV in Japan are likely associated with the popularization of eating pork.

The 3e strains have been isolated from HEV-infected humans and pigs in restricted areas of Japan, including Hokkaido, Miyagi, Tochigi, Mie and Ehime. Of note, although the HEV strain (HE-JA11-1701) identified in the hepatitis patient from Mie in the present study was segregated into subgenotype 3e, it differed from the six previously identified Mie 3e strains by 12.9–13.9%, from all other reported 3e strains in Japan by 8.2–12.9%, and from European strains (22 strains in Italy, France, Germany, Hungary and the UK, retrievable from the DNA Data Bank of Japan/GenBank/European Molecular Biology Laboratory databases as of March 2013) by 7.9–12.2% within the overlapping 304–411-nt sequence, suggesting that the HE-JA11-1701 strain is remotely related to the 3e strains reported thus far both in Japan and European countries. Interestingly, however, the HE-JA11-1701 strain was found to be 99.8% identical to the boar strain (JBOAR012-Mie08) recovered from a wild boar in Mie, suggesting that both strains may have been generated from a common ancestor 3e strain.

In 2003, the first case of hepatitis E following the ingestion of uncooked boar liver was reported.[15] Subsequently, many other sporadic cases of acute hepatitis E developing after the consumption of cooked, undercooked or uncooked meat/viscera of wild boars have been reported in Japan.[16,17,20,21,31] A small outbreak of

acute hepatitis E caused by the simultaneous ingestion of wild boar meat at a barbecue party has also been reported,[32] where 12 persons exclusively ate charcoal-grilled, but partially undercooked, wild boar meat. Li *et al.*[14] provided direct evidence of zoonotic food-borne transmission of HEV from a wild boar to a human, by investigating a case of hepatitis E acquired after the individual ate wild boar meat. Genotype 3 HEV RNA was detected in both the patient's serum and the leftover wild boar meat. The infectivity of HEV in boar liver has been demonstrated by culturing HEV in the liver homogenate of HEV-infected wild boars in PLC/PRF/5 and A549 cells.[33]

Although we were unable to provide direct evidence that the boar was the source of infection because there were no leftovers, it seems reasonable to speculate that our patient developed acute hepatitis E via the consumption of meat/viscera from a wild boar based on the following: (i) the patient contracted acute hepatitis E after a reasonable period of time (approximately 2 months) after he ate the meat/viscera of a wild boar that he had captured himself as a hunter; (ii) the HEV strain that was recovered from the patient exhibited high genomic similarity (99.8%) to the HEV strain obtained from a wild boar that was captured near the patient's hunting area; and (iii) the HEV strains obtained from both the hepatitis patient and the wild boar were segregated into a rare subgenotype (3e) and were far from the 3e strains reported in Japan as well as European countries, including Italy, France, Germany, Hungary and the UK.

The previously identified Mie 3e strains are unique in that they are closely related mutually to form a cluster, supported by a bootstrap value of 71% (Fig. 2), with a nucleotide difference of only 0.2–1.5%. However, the infectious source(s) or even risk factor(s) for HEV infection in these six hepatitis cases remain unclear.[27,34] Our previous study indicated that swine 3e HEV strains obtained from pigs within the same herd exhibited 97.1–100% nucleotide sequence identities, suggesting that a predominant 3e strain unique to a herd making an inroad into the herd in the past has been maintained over a long period in the herd.[22] The periodic occurrence of HEV infection by homologous 3e strains during the long period of 2004–2012 suggests the maintenance of the same infectious source(s) in a particular region(s), such as a swine farm in Mie prefecture. Investigating the prevalence of HEV infection in a large number of wild boars would help to clarify whether the dominant 3e HEV strains are circulating among a herd(s) of boars in the wild in Mie prefecture. Although HEV is now acknowledged to have a nationwide distribution in Japan as an agent responsible for hepatitis, its transmission route remains obscure in most cases.[4] Further efforts to clarify the sources and routes of HEV infection in Japan are therefore warranted.

In conclusion, the close genetic relationship (99.8%) observed between the HE-JA11-1701 strain obtained from a hunter with sporadic acute hepatitis E who had consumed wild boar meat before disease onset and the JBOAR012-Mie08 strain recovered from a wild boar captured near the patient's hunting area strongly supports the zoonotic transmission of HEV from a wild boar to the patient as the cause of the clinical HEV infection. The use of molecular epidemiological approaches would be helpful for further elucidating the infectious sources and routes of HEV in patients with hepatitis E in Japan, where the number of reported autochthonous hepatitis E cases has been increasing, and the transmission source of HEV remains unknown in most cases.

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Hepatology Research

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Original Article

Full genome analysis of Philippine indigenous subgenotype IA hepatitis A virus strains from Japanese patients with imported acute hepatitis A

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Aim: Hepatitis A virus (HAV) is the most common cause of infectious hepatitis worldwide. Although hepatitis A cases imported from South-East Asian countries, including the Philippines, have been reported in Japan, the molecular epidemiological data have been limited for these HAV-endemic countries.

Methods: The full-length genomic sequences of HAV isolates were determined and subjected to the phylogenetic analyses.

Results: The HAV isolates (HA12-0796 and HA12-0938) obtained from two Japanese patients who developed acute hepatitis A in July 2012, 1 month after traveling to the Philippines, where they consumed undercooked shellfish, differed by only one nucleotide (nt) over the entire genome. These HAV isolates of genotype IA were 99.1–99.5% identical within 228–237 nt to those recovered from river water in the Philippines, suggesting that the HA12-0796 and HA12-0938 isolates represent HAV circulating in the Philippines. HAV isolates belonging to one of the two IA sublineages (IA-2) which were implicated in some of the mini-epidemics in 2010 in Japan are hypothesized to be connected with the Philippines. In support of this speculation, the present IA isolates (HA12-0796 and HA12-0938) shared 98.8% identity over the entire genome with one IA-2 isolate (HAJIH-Fukuo10) recovered from a Japanese female who developed a domestic HAV infection during the mini-epidemics. 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%.

Conclusion: These results indicate that HAV isolates belonging to the IA-2 lineage might have been imported from the Philippines.

Key words: complete genome, hepatitis A virus, hepatitis A, Philippines, phylogenetic analysis

INTRODUCTION

HEPATITIS A VIRUS (HAV) causes acute hepatitis in humans worldwide, and is transmitted largely through fecal–oral contamination, often via contaminated food, drink or objects handled by infected persons, but is rarely transmitted sexually or parenterally.[1,2] Infection with HAV is endemic in developing countries, and the majority of individuals in these countries are exposed to HAV during childhood. In contrast, the adult population in industrialized countries, including Japan, has had a decreasing exposure rate to HAV due to improvements in hygiene and sanitation conditions.[3] Young children are usually asymptomatic, although the likelihood of symptoms tends to increase with age. In older children and adults, there is a wide range of clinical manifestations from mild, anicteric infection, to severe, fulminant hepatic failure.[2]

Hepatitis A virus is the only member of the genus *Hepatovirus* within the family *Picornaviridae*,[4] and its viral genome consists of a 7.5-kb, positive-stranded RNA with a single long open reading frame (ORF). The ORF of 2227 amino acids (a.a.) is organized into three functional regions termed P1, P2 and P3. P1 encodes the capsid polypeptides VP1–VP4, whereas P2 and P3

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encode non-structural polypeptides. The ORF is preceded by a 5′-untranslated region (UTR) and is followed by a 3′-UTR with a short poly(A) tail.[2] Despite this antigenic uniformity with a single serotype, HAV has been shown to display a modest degree of genetic diversity. The HAV strains have been classified into six genotypes (I–VI), of which genotypes I, II and III are found in humans. These are further divided into subgenotypes IA and IB, IIA and IIB, and IIIA and IIIB, respectively.[5] Genotype I is most prevalent worldwide, and subgenotype IA is more common than IB. Currently, the entire or nearly entire nucleotide sequence is available for at least 50 human HAV isolates of subgenotypes IA, IB, IIA, IIB, IIIA and IIIB,[5–11] but for little or no isolates in many developing countries where HAV is endemic.

In many Asian countries, the improved hygiene standards and socioeconomic conditions have led to a reduction in exposure to HAV in childhood. However, the persistence of circulating HAV may lead to hepatitis A outbreaks, particularly in adolescents and adults. In other countries and specific areas where socioeconomic conditions have not improved as markedly, HAV endemicity remains medium to high. For example, little is known about the genomic characteristics of the HAV circulating in the Philippines, where HAV remains highly endemic, although region-dependent variations are suggested.[12–14] We recently encountered two Japanese patients who developed acute hepatitis A 1 month after traveling to the Philippines and were presumed to have contracted HAV infection through the consumption of undercooked shellfish while staying there. The present study was conducted to characterize the entire genomic sequence of the HAV isolates recovered from the two patients, most likely representing a prototype HAV strain circulating in the Philippines, in an attempt to gain further insight into the molecular epidemiology of HAV infection in Japan, where mini-epidemics of HAV in 2010 were suggested to have been caused in part by a IA sublineage (IA-2) HAV with a hypothesized Philippine connection.[15]

METHODS

Patients

A 46-YEAR-OLD JAPANESE female (patient 1) living in Tochigi Prefecture, Japan, presented to our hospital with complaints of a high fever, anorexia, vomiting and abdominal pain on 13 July 2012, 4 days after the onset of the illness, and was hospitalized with a diagnosis of acute hepatitis. Upon admission, the patient had an elevated alanine aminotransferase (ALT) level of 4491 IU/L, elevated aspartate aminotransferase (AST) level of 7353 IU/L and slight bilirubinemia, with a bilirubin level of 1.5 mg/dL. The abnormal bilirubin levels persisted until 15 days after admission, with the highest level of 5.8 mg/dL observed on the 8th and 11th hospital day (Table 1). She was negative for serum markers of hepatitis B, C and E viruses and the immunoglobulin (Ig)M class of antibodies against Epstein–Barr virus and cytomegalovirus, but positive for IgM-class antibodies to HAV (anti-HAV) by a chemiluminescent immunoassay (Architect; Abbott Japan, Tokyo, Japan). Based on the evidence that she had ingested undercooked shellfish (bivalves) while traveling on Leyte Island in the Philippines 1 month before the onset of the disease, she was diagnosed with imported acute hepatitis A. During her admission, she told us that her friend, patient 2 (a 44-year-old Japanese male) who visited the same place and consumed undercooked shellfish in the same restaurant with her, was admitted to a general hospital 4 days after her admission.

Patient 2, who lived in Kanagawa Prefecture, Japan, had experienced diarrhea, a high fever, general malaise and appetite loss starting on 10 July 2012, and visited a nearby Internal Medicine Clinic 7 days after the onset of the illness. On that day, he was transferred to a general hospital in Kanagawa and was hospitalized with a clinical diagnosis of acute hepatitis. A physical examination on admission was essentially normal, except for jaundice. His laboratory data at admission showed an elevated ALT level of 4523 IU/L, AST level of 3396 IU/L and bilirubin level of 5.5 mg/dL. The abnormal bilirubin levels persisted until 7 days after admission, with the highest level of 6.4 mg/dL observed on day 4 (Table 1). His prothrombin time was 48% on admission, and increased thereafter. Based on his positivity for IgM anti-HAV antibodies and a history of eating undercooked shellfish during his trip to the Philippines, he was also diagnosed with imported acute hepatitis A.

After admission, patients 1 and 2 both recovered rapidly without sequelae and were discharged on the 15th and 11th hospital days, respectively. With informed consent, one or three serum samples were obtained from each patient and stored at −80°C until they were analyzed.

Detection of HAV RNA

Serum samples were assayed for HAV RNA by reverse transcription polymerase chain reaction (RT–PCR) with nested primers derived from the well-conserved area of the 5′-UTR of the HAV genome according to the previously described method, with slight modifications.[16]

Table 1 Laboratory parameters and detection of HAV RNA in serum samples obtained periodically from two patients with acute hepatitis A

Days after onset (hospital day)	AST (IU/L)	ALT (IU/L)	Total bilirubin (mg/dL)	Prothrombin time (%)	HAV RNA
Patient 1					
4 (1)	7353	4491	1.5	46	+
5 (2)	6101	5335	2.5	50	
6 (3)	3812	4848	2.8		
8 (5)	1304	3005	4.1	70	
9 (6)	697	2315	4.2	73	
10 (7)	324	1577	5.6	75	
11 (8)	226	1179	5.8	75	
14 (11)	104	527	5.8	86	
16 (13)	55	294	3.2	88	
18 (15)	43	170	3.2		
37	29	33	1.0	99	+
100	22	15	0.5		-
Patient 2					
7 (1)	3396	4523	5.5	48	
10 (4)	761	2060	6.4	81	
13 (7)	212	820	2.5	103	+
17 (11)	86	323	1.6	103	
25	57	130	1.1	108	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HAV, hepatitis A virus.

Briefly, the RNA extracted from 100 μL of serum were subjected to cDNA synthesis with reverse transcriptase (Superscript II; Life Technologies, Tokyo, Japan) and the HA009 primer (5′-TCA ATG CAT CCA CTG GAT GAG-3′). The cDNA were subjected to first-round PCR with *Ex Taq* DNA polymerase (TaKaRa Bio, Shiga, Japan) and the HA018 (5′-GAT ACC TCA CCG CCG TTT GC-3′) and HA009 primers for 35 cycles (94°C for 2 min before the start of cycling: 94°C, 30 s; 55°C, 30 s; 72°C, 90 s [additional 7 min in the last cycle]). The second-round PCR for 25 cycles was carried out under the same conditions as the first-round PCR except for extension for 60 s with primers HA019 (5′-CGT TTG CCT AGG CTA TAG GCT-3′) and HA020 (5′-CAG TCC TYC GGC GTT GAA TGG-3′ [Y = T or C]). The amplification product of the first-round PCR was 474 bp, and that of the second-round PCR was 437 bp. The PCR product of the second-round PCR was subjected to electrophoresis on an agarose gel, and a sample with a visible band at 437 bp was considered to be positive for HAV RNA.

Amplification of the full-length HAV genome

Total RNA was extracted from 100 or 500 μL of serum samples, and was subjected to cDNA synthesis, followed by nested PCR of four overlapping regions in the central portion of the HAV genome, excluding the extreme 5′- and 3′-terminal regions, using enzymes (KOD-plus [Toyobo, Osaka, Japan], *LA Taq* DNA polymerase [TaKaRa Bio] or *Ex Taq* DNA polymerase) and primers whose sequences were derived from well-conserved areas across six subgenotypes (IA, IB, IIA, IIB, IIIA and IIIB),[10] as well as those obtained during the amplification procedure. The amplified regions were nt 47–153 (107 nt) (primer sequences excluded), nt 96–3418 (3323 nt), nt 3375–5969 (2595 nt) and nt 5383–6609 (1227 nt), where the nucleotide numbers are in accordance with the HA12-0796 isolate from patient 1, whose entire nucleotide sequence was determined in the present study. cDNA covering the extreme 5′-end sequence (nt 1–61) were tailed with dGTP homopolymer by a terminal deoxynucleotidyl transferase (New England BioLabs, Tokyo, Japan), and amplified by single-sided PCR with 41-mer oligonucleotides containing $(C)_{15}$ according to the method described previously.[17] Amplification of the 3′-end sequence (nt 6289–7477 [1189 nt], excluding the poly(A) tail) was attempted by a modified rapid amplification of cDNA ends technique as described previously.[18]

Sequence analysis

The amplification products were purified using a FastGene Gel/PCR Extraction kit (NIPPON Genetics, Tokyo, Japan) and then both strands were sequenced directly by employing an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems). The extreme 5′- and 3′-end sequences were determined based on the consensus sequence of at least five clones, which were obtained after being inserted into the T-Vector, pMD20 (TaKaRa Bio). The sequence analysis was performed using the Genetyx software program (ver. 11.0.4; Genetyx, Tokyo, Japan), and multiple alignments were generated by the CLUSTAL W software program (ver. 2.1).[19] Phylogenetic trees were constructed by the neighbor-joining method,[20] using the Kimura two-parameter correction model and 1000 replicates of bootstrap re-sampling as implemented in the MEGA 5 software program (ver. 5.1.0).[21] The full-length nucleotide sequences of 50 HAV isolates (see Fig. 1 for accession numbers) and VP1/2A region sequences (220 nt) of 78 HAV isolates (see Fig. 2 for accession numbers) that were retrievable from DNA Data Bank of Japan (DDBJ)/GenBank/European Molecular Biology Laboratory (EMBL) databases as of January 2013 were included for comparison. The nucleotide sequence data reported in this study have been assigned DDBJ/GenBank/EMBL accession numbers AB793725–AB793726 for two entire HAV genomes.

RESULTS

Characterization of the full-length HAV genomes

STORED SERUM SAMPLES obtained from patient 1 on the 4th and 37th days and from patient 2 on the 13th day after the disease onset were positive for HAV RNA, which was detected by nested RT–PCR with primers targeting the 5′-UTR (Table 1). The full-length genomic sequence was determined for HAV in the serum samples from both patient 1 (HA12-0796: the first hospital day) and patient 2 (HA12-0938: the seventh hospital day).

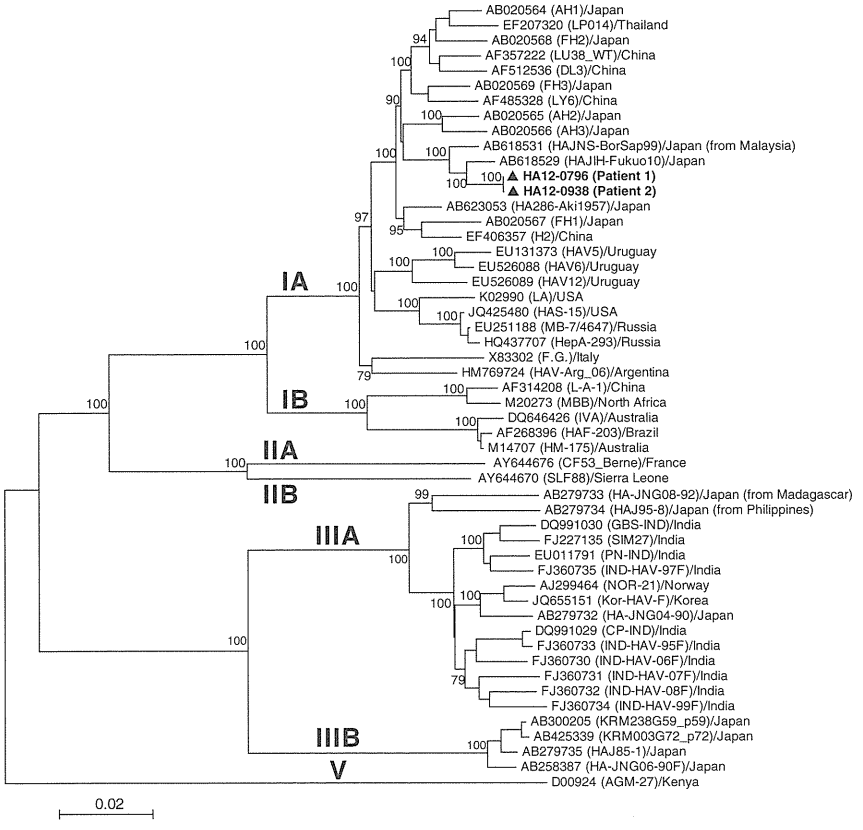

Figure 1 Phylogenetic tree constructed based on the full-length nucleotide sequences of 52 hepatitis A virus (HAV) isolates by the neighbor-joining method using AGM-27 (D00924) as an outgroup. In addition to the HA12-0796 and HA12-0938 isolates of subgenotype IA obtained in the present study, which are indicated in bold for visual clarity, 50 reported HAV isolates of genotypes/subgenotypes IA, IB, IIA, IIB, IIIA, IIIB and V whose entire or nearly entire sequences are known were included for comparison. The bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 re-samplings.

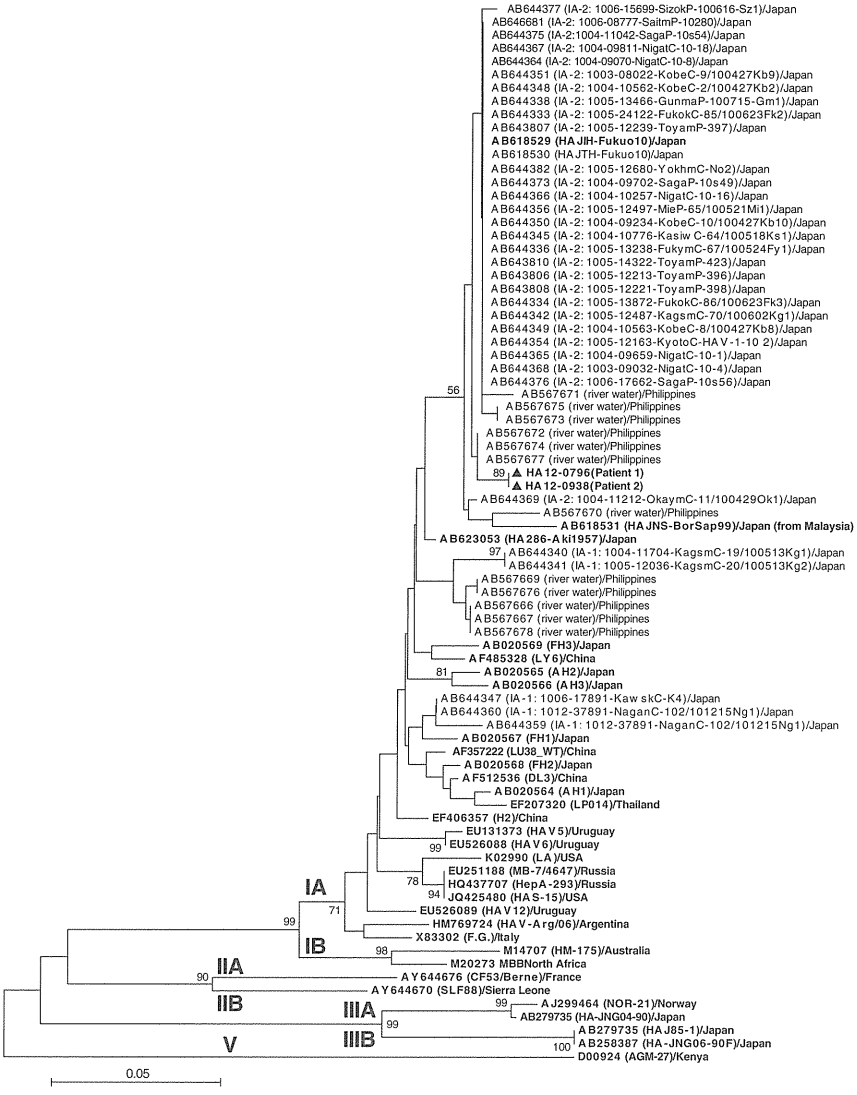

Figure 2 Phylogenetic tree constructed by the neighbor-joining method using AGM-27 (D00924) as an outgroup, based on the partial nucleotide sequences of the VP1/2A region (220 nt). In addition to the HA12-0796 and HA12-0938 isolates obtained in the present study, IA isolates (IA-1 and IA-2 sublineages and others) identified during the mini-epidemics in 2010 in Japan[15] and those recovered from river water in the Philippines, as well as representative hepatitis A virus (HAV) isolates of genotypes/subgenotypes IA, IB, IIA, IIB, IIIA and IIIB whose entire genomic sequences are known were included for comparison. The HAV isolates with known entire genomic sequence are indicated in bold for visual clarity. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 re-samplings.

The HA12-0796 and HA12-0938 isolates had a genomic length of 7477 nt excluding the poly(A) tract at the 3′-terminus, and possessed a single long ORF of 6681 nt (nt 734–7414) that encoded a polyprotein of 2227 a.a. The single ORF was divided into three functional regions termed P1 (2370 nt), P2 (1893 nt) and P3 (2418 nt) and their subregions as reported previously.[7,22] HA12-0796 and HA12-0938 differed from each other by only one nucleotide (at nt 2164; the third letter of codon 477) over the entire genome, and were 100% identical in the amino acid sequence of the polyprotein. Comparisons of the HA12-0796 and HA12-0938 genomes against reported HAV genomes of genotypes I, II, III and V whose entire or nearly entire nucleotide sequences were known (see Fig. 1), revealed that HA12-0796 and HA12-0938 were most closely related to the human HAV isolates of subgenotype IA ($n = 23$), with identities of 94.3–98.8% over the entire genome, and were 90.5–91.2% similar to the human HAV isolates of subgenotype IB ($n = 5$) and only 81.4–86.5% similar to the human HAV isolates of genotypes II and III and a simian HAV isolate of genotype V ($n = 22$). The phylogenetic tree constructed by the neighbor-joining method based on the full genomic sequence confirmed that HA12-0796 and HA12-0938 segregated into subgenotype IA, with a bootstrap value of 100% (Fig. 1). Of note, HA12-0796 and HA12-0938 formed a cluster with HAJIH-Fukuo10 (AB618529) that