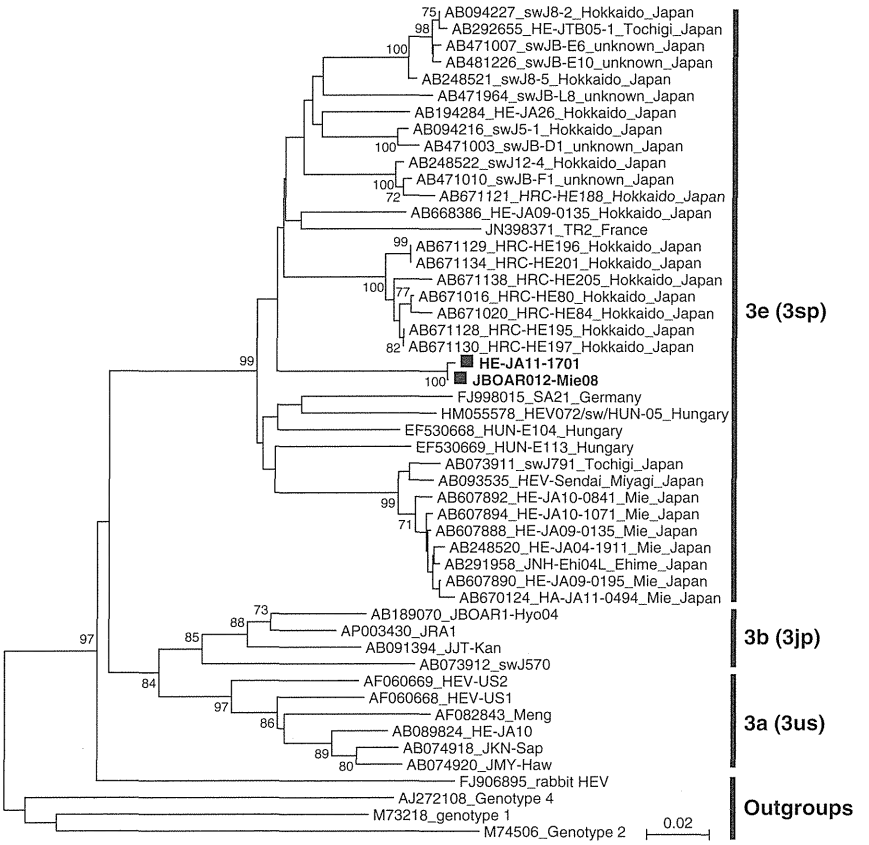

Figure 2 A neighbor-joining tree of the 412-nucleotide (nt) open reading frame 2 (ORF2) sequences of the two hepatitis E virus (HEV) isolates obtained in this study with 44 reference sequences of genotype 3 and the outgroup isolates. The reference sequences are shown with accession numbers, followed 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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Original Article

Characterization of sporadic acute hepatitis E and comparison of hepatitis E virus genomes in acute hepatitis patients and pig liver sold as food in Mie, Japan

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Aim: To characterize hepatitis E in Mie prefecture and to investigate whether raw pig liver sold as food in Mie is contaminated with hepatitis E virus (HEV) strains similar to those recovered from patients.

Methods: Seventeen patients with sporadic acute hepatitis E treated from 2004 to 2012 were studied. A total of 243 packages of raw pig liver from regional grocery stores were tested for the presence of HEV RNA. The partial genomic sequences of human and swine HEV isolates were determined and subjected to the phylogenetic analyses.

Results: The HEV isolates recovered from the 17 patients segregated into genotype 3 (n = 15) and genotype 4 (n = 2), and 15 genotype 3 isolates further segregated into 3e (n = 11) and 3b (n = 4). Pig liver specimens from 12 (4.9%) of the 243 packages had detectable HEV RNA. All 12 swine HEV isolates were grouped into genotype 3 (3a or 3b). Although no 3e strains were isolated from pig liver specimens, two 3b swine strains were 99.5–100% identical to two HEV strains recovered from hepatitis patients, within 412-nt partial sequences.

Conclusion: The 3e HEV was prevalent among hepatitis E patients. HEV RNA was detected in approximately 5% of pig liver sold as food. The presence of identical HEV strains between hepatitis patients and pig liver indicated that pigs play an important role as reservoirs for HEV in humans in Mie. Further studies are needed to clarify the source of 3e HEV in the animal and environmental reservoirs.

Key words: genotype, hepatitis E virus, hepatitis E, phylogenetic analysis, pig liver

INTRODUCTION

HEPATITIS E, AN important human disease caused by the hepatitis E virus (HEV), is characterized by epidemics or explosive outbreaks of acute hepatitis. Hepatitis E is endemic to many resource-limited regions of the world, and sporadic and cluster cases of hepatitis E are observed in industrialized countries, most likely via zoonotic infection.[1,2]

Hepatitis E virus is classified as a *Hepevirus* in the family Hepeviridae.[3] The genome of HEV is a single-stranded, positive sense RNA composed of 7.2 kb, and possesses a short 5′-untranslated region (UTR), followed by three open reading frames (ORF: ORF1, ORF2 and ORF3) and then a short 3′-UTR.[4] HEV is a virus that is capable of replicating efficiently in established human cell lines such as PLC/PRF/5 and A549.[5] At least four genotypes of HEV infecting humans are recognized as a species, each dominant in a given geographic area.

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Genotype 1 includes strains from Asia and Africa; genotype 2 includes a Mexican strain and few variants from Africa; genotype 3 includes human and animal HEV strains distributed widely throughout the world; and genotype 4 includes human and animal HEV strains distributed mainly in Asian countries, including China and Japan.[6] 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 (3 us) and 3e (3sp), where "jp" stands for Japan-type, "us" for US-type and "sp" for Spanish (European) type.[7–9]

Hepatitis E is considered to be as a zoonotic disease,[10–12] and animals such as domestic pigs and wild boars are important reservoirs for HEV.[11–13] Sporadic and cluster cases of acute hepatitis E due to the consumption of raw or undercooked pig livers have been reported in Japan.[14] It has previously been shown that approximately 2% of the pig livers sold in local grocery stores in Hokkaido, Japan,[11] and 11% in the USA[15] were positive for swine HEV RNA.

Our previous studies[16,17] suggested that European-type subgenotype 3e HEV strains that are rare in Japan are predominant in the sporadic cases of acute hepatitis E in Mie prefecture, located in the central region of Japan, although their source/route of HEV infection remains largely unknown. The present study was conducted to characterize the hepatitis E cases diagnosed in Mie from 2004 to 2012, and to identify the HEV strains in raw pig liver sold as food purchased in grocery stores in the area where the patients lived in an attempt to clarify whether the swine HEV strains are phylogenetically associated with those from hepatitis E patients in Mie.

Figure 1 Map of Japan showing two prefectures (Mie and Aichi) and a magnified map of Mie prefecture showing the four cities where hepatitis E patients were identified in the present study. One (no. 11) of the 17 patients studied ingested meat from a wild boar in Aichi prefecture.

METHODS

Sera from patients with sporadic cases of acute hepatitis E

SERUM SAMPLES WERE obtained from 17 patients at admission who were seen at five university or city hospitals in Mie (Fig. 1), with a final clinical diagnosis of sporadic acute hepatitis E (see Table 1). These patients were admitted to the respective hospitals between July 2004 and July 2012, and each patient was from the same geographic region where the respective hospital was located, except for patient (no. 11) who lived in Aichi but received care at a city hospital in Suzuka city, Mie. They were all negative for the immunoglobulin (Ig)M class of antibodies against hepatitis A virus (anti-HAV IgM), hepatitis B virus (HBV) markers (anti-HBV core IgM and hepatitis B surface antigen [HBsAg]), anti-hepatitis C virus (anti-HCV) and IgM class antibodies against Epstein–Barr virus and cytomegalovirus. The presence of anti-HAV IgM, anti-HBV core IgM, HBsAg and anti-HCV was examined using commercially available kits (Abbott Japan, Tokyo, Japan). Among the 17 patients, seven patients (patients 1–6, 8 and 9) have been described in our previous studies.[16,17] The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of the institutions. Informed consent was obtained from each patient.

Pig liver specimens

A total of 243 packages of raw pig liver that were for sale as food, were purchased from 16 grocery stores in Mie between July 2011 and March 2013, and sent to Jichi Medical University for detection of HEV RNA as described below. Pig liver packages purchased were local products in Mie, while most packages purchased in

Table 1 Characteristics of patients with sporadic acute hepatitis E in Mie, Japan

Patient no.†	Age (years)	Sex	Residence (city)	Year of onset	Peak ALT (IU/L)	Peak AST (IU/L)	Peak T-Bil (mg/dL)	Lowest PT%	IgG anti-HEV (OD_{450})‡	IgM anti-HEV (OD_{450})‡	IgA anti-HEV (OD_{450})‡	HEV RNA (copies/mL)‡
1	51	M	Tsu	2004	5150	8220	7.6	30	0.101 (−)	>3.000 (+)	0.842 (+)	1.2×10^7
2	54	M	Kuwana	2005	4735	4535	5.5	80	1.315 (+)	2.169 (+)	>3.000 (+)	6.2×10^4
3	58	M	Suzuka	2007	2791	1647	8.7	87	>3.000 (+)	>3.000 (+)	>3.000 (+)	6.6×10^4
4	55	M	Yokkaichi	2007	4849	2986	5.1	70	1.687 (+)	>3.000 (+)	2.725 (+)	7.4×10^4
5	46	M	Suzuka	2008	4722	4070	12.0	58	2.729 (+)	2.074 (+)	>3.000 (+)	2.8×10^4
6	61	M	Suzuka	2009	1560	2023	1.4	54	1.165 (+)	>3.000 (+)	2.736 (+)	1.5×10^5
7	67	M	Tsu	2010	1218	616	0.6	100	0.513 (+)	1.568 (+)	1.833 (+)	4.0×10^4
8	67	M	Suzuka	2010	2115	1684	6.2	82	1.586 (+)	2.704 (+)	2.443 (+)	1.2×10^8
9	66	M	Suzuka	2011	6221	5540	19.2	39	0.820 (+)	2.600 (+)	1.783 (+)	7.3×10^4
10	40	M	Yokkaichi	2011	2295	2593	3.8	11	>3.000 (+)	>3.000 (+)	>3.000 (+)	4.0×10^4
11	63	M	Kariya§	2012	456	171	0.6	93	1.774 (+)	2.185 (+)	>3.000 (+)	1.5×10^5
12	36	F	Suzuka	2012	1154	437	0.7	87	1.146 (+)	2.592 (+)	2.494 (+)	4.2×10^3
13	61	M	Suzuka	2012	525	141	2.4	82	1.541 (+)	1.342 (+)	2.799 (+)	5.7×10^2
14	68	M	Suzuka	2012	375	83	1.7	93	2.725 (+)	2.362 (+)	>3.000 (+)	(+) <10
15	77	M	Suzuka	2012	516	337	1.0	105	>3.000 (+)	1.772 (+)	2.607 (+)	6.2×10^2
16	64	M	Yokkaichi	2012	1928	1577	3.8	23	2.256 (+)	2.862 (+)	2.679 (+)	9.1×10^2
17	61	M	Suzuka	2012	918	542	2.0	102	1.604 (+)	1.525 (+)	2.623 (+)	8.1×10^4

†Patients 1, 5, 6 and 8 correspond to cases 1–4 in the previous report by Okano *et al.*,[16] respectively, and patients 1–6, 8 and 9 correspond to cases 2 and 6–12 in the previous report by Nakano *et al.*,[17] respectively.
‡Detected in serum samples obtained at the first visit.
§Kariya is located in Aichi prefecture.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HEV, hepatitis E virus; Ig, immunoglobulin; OD, optical density; PT, prothrombin time; T-Bil, total bilirubin.

Yokkaichi city were from Aichi, the neighboring prefecture (see Table 3 for detail). The pig liver in each package had been processed into slices or a block of 52–1892 g (mean, 367 g), and two to 34 packages were available from each store (mean, 15.2 packages). Several pieces of tissue specimens (~5 g in total) were obtained from each package and stored at −80°C until testing.

ELISA for detecting anti-HEV antibodies

To detect anti-HEV IgG, IgM and IgA, enzyme-linked immunosorbent assays (ELISA) using human serum samples were performed using purified recombinant ORF2 protein of the HEV genotype 4 that had been expressed in the pupae of silkworms,[18] as described previously.[19] The optical density (OD) of each sample was read at 450 nm. The cut-off value used for the anti-HEV IgG assay was 0.175, that for the anti-HEV IgM assay was 0.440 and that for the anti-HEV IgA assay was 0.642. Test samples with OD values for anti-HEV IgG, IgM and IgA equal to or greater than the respective cut-off value were considered to be positive for anti-HEV.

Detection of HEV RNA in serum samples and liver tissue samples

Total RNA was extracted from 100 μL of human serum using the TRIZOL-LS reagent (Life Technologies, Carlsbad, CA, USA) and was dissolved in 10 μL of nuclease-free distilled water. For the pig livers, a piece of pig liver (100 mg) was minced with a razor blade and homogenized with a BioMasher II (Nippi Incorporated, Tokyo, Japan), and the total RNA was extracted from the liver homogenate using the TRIZOL reagent (Life Technologies) and was dissolved in 100 μL of nuclease-free distilled water. The RNA preparation thus obtained (10 μL) was reverse transcribed with SuperScript II (Life Technologies), and subsequent nested polymerase chain reaction (ORF2-457 PCR) was performed with primers derived from the areas of the ORF2 region that are well-conserved across all four genotypes, using the method described previously.[18] The size of the amplification product of the first-round PCR was 506 bp, and that of the amplification product of the second-round PCR was 457 bp. The PCR product of the second-round PCR was subjected to electrophoresis on an agarose gel, and a sample with a visible band at 457 bp was considered to be positive for HEV RNA.

To confirm the presence of HEV RNA, another nested reverse transcription (RT)–PCR (ORF1-459 PCR) with primers targeting the 5′-UTR and 5′-terminus of the ORF1 region,[18] capable of amplifying all four known genotypes of HEV strains reported thus far, was carried out. The size of the amplification product of the first-round PCR was 567 bp, and that of the amplification product of the second-round PCR was 459 bp. The specificity of the two RT–PCR assays was verified by a sequence analysis as described below. The sensitivity of the RT–PCR assay was assessed as described previously.[8,18]

To avoid contamination during the PCR procedures, the guidelines established by Kwok and Higuchi[20] were strictly observed.

Quantitation of HEV RNA

Hepatitis E virus RNA was quantitated by real-time detection via RT–PCR according to a method described previously[21] with slight modifications, using a culture supernatant containing a known amount of HEV progeny (genotype 3; 1.2×10^7 copies/mL) as a standard. The load of the standard HEV was determined using an *in vitro*-transcribed RNA standard.[22] In brief, total RNA was extracted from 2–100 μL of serum or liver homogenate using TRIZOL-LS or TRIZOL and was subjected to real-time RT–PCR with a QuantiTect Probe RT–PCR Kit (QIAGEN, Tokyo, Japan), using primers and a probe with a 5′-reporter dye (FAM) and a 3′-quencher dye (TAMRA) targeting the well-conserved ORF3 region using a LightCycler apparatus (Roche Diagnostic, Tokyo, Japan). The thermal cycler conditions were 50°C for 20 min during stage 1, 95°C for 15 min during stage 2, and 45 cycles of 95°C for 1 s and 60°C for 60 s during stage 3. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate, and the mean value was adopted for subsequent analyses.

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 Japan, Tokyo, Japan) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Japan). The sequence analysis was performed using the Genetyx software program (version 11.0.4; Genetyx, Tokyo, Japan). Phylogenetic analyses were conducted by the neighbor-joining method based on the 412-nt ORF1 or 412-nt ORF2 sequence with 1000 bootstrapping replicates, using the MEGA 5 software program (version 5.2.0).[23] The nucleotide sequence data determined in this study have been deposited in the DNA Data Bank of Japan/European

Molecular Biology Laboratory/GenBank databases under accession numbers AB824672–AB824712.

RESULTS

Characteristics of the 17 patients with sporadic acute hepatitis E

OF THE 17 patients studied, all but one were male. The age of the patients ranged 36–77 years, with a mean age of 58.6 years. Four patients (patients 1, 9, 10 and 16) developed a severe form of acute hepatitis E, with a lowest prothrombin time of less than 40% (unaccompanied by hepatic encephalopathy), and had a peak total bilirubin (T-Bil) level of 3.8–19.2 mg/dL, a peak alanine aminotransferase (ALT) level of 1928–6221 IU/L and a peak aspartate aminotransferase (AST) level of 1577–8220 IU/L (Table 1). Among the remaining 13 patients with acute hepatitis E, five patients had an elevated T-Bil level of more than 5.0 mg/dL and eight patients had elevated ALT and/or AST levels of more than 1000 IU/L. Despite the marked elevation in the T-Bil, ALT and AST levels at the initial examination, the abnormal liver function test values normalized rapidly within 1 month in all 17 patients. The acute phase sera from all 17 patients were positive for anti-HEV IgM, with the OD values ranging from 1.342 to more than 3.000, and for anti-HEV IgA, with the OD values ranging from 0.842 to more than 3.000, while the anti-HEV IgG was positive in 16 patients. In the remaining patient (no. 1), anti-HEV IgG became positive (OD, 2.477) 7 days after the initial examination. All 17 patients had detectable HEV RNA in the serum samples obtained during the acute phase, including those obtained at admission, with the virus load ranging from less than 10 to 1.2×10^8 copies/mL. The HEV isolates obtained from 15 patients were of genotype 3, while those from the remaining two patients were of genotype 4.

Possible risk factors for acquiring hepatitis E

Among the 17 patients studied, one patient (no. 3) had a history of traveling to China, where the patient consumed raw vegetables and sushi (raw fish and shellfish) and drank unboiled water 1 month before the onset of the disease, and was diagnosed with imported hepatitis E (Table 2), supported by phylogenetic analysis of the isolated HEV strain (see below). The remaining 16 patients had no history of travel outside Japan, or any

Table 2 HEV isolates and possible sources of HEV infection in patients with sporadic acute hepatitis E in Mie, Japan

Patient no.†	Name of HEV isolate	HEV genotype/ subgenotype	Possible source of infection
1	HE-JA04-1911	3e	Intestine from pig or cow, raw shellfish and unboiled water
2	HE-JA05-0753	3b	Raw liver from pig or cow
3	HE-JA07-0229	4	Imported from China (raw vegetables, raw fish and unboiled water)
4	HE-JA10-0841	3e	Liver from a pig
5	HE-JA09-0135	3e	Unknown‡
6	HE-JA09-0195	3e	Unknown‡
7	HE-JA11-1701	3e	Meat/viscera from a wild boar
8	HE-JA10-1071	3e	Unknown‡
9	HE-JA11-0494	3e	Unknown
10	HE-JA11-0975	3b	Raw shellfish
11	HE-JA12-0202	4	Liver from a wild boar in Aichi prefecture
12	HE-JA12-0394	3e	Unknown‡
13	HE-JA12-0483	3b	Unknown‡
14	HE-JA12-0546	3e	Unknown‡
15	HE-JA12-0647	3e	Unknown‡
16	HE-JA12-0752	3e	Barbecued pork
17	HE-JA12-0940	3b	Raw meat from a horse

†Patients 1, 5, 6 and 8 correspond to cases 1–4 in the previous report by Okano *et al.*,[16] respectively, and patients 1–6, 8 and 9 correspond to cases 2 and 6–12 in the previous report by Nakano *et al.*,[17] respectively.
‡Except for patient 9, seven patients with unknown sources of infection reported consumption of raw fish (sashimi and/or sushi) before the disease onset.
HEV, hepatitis E virus.

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.

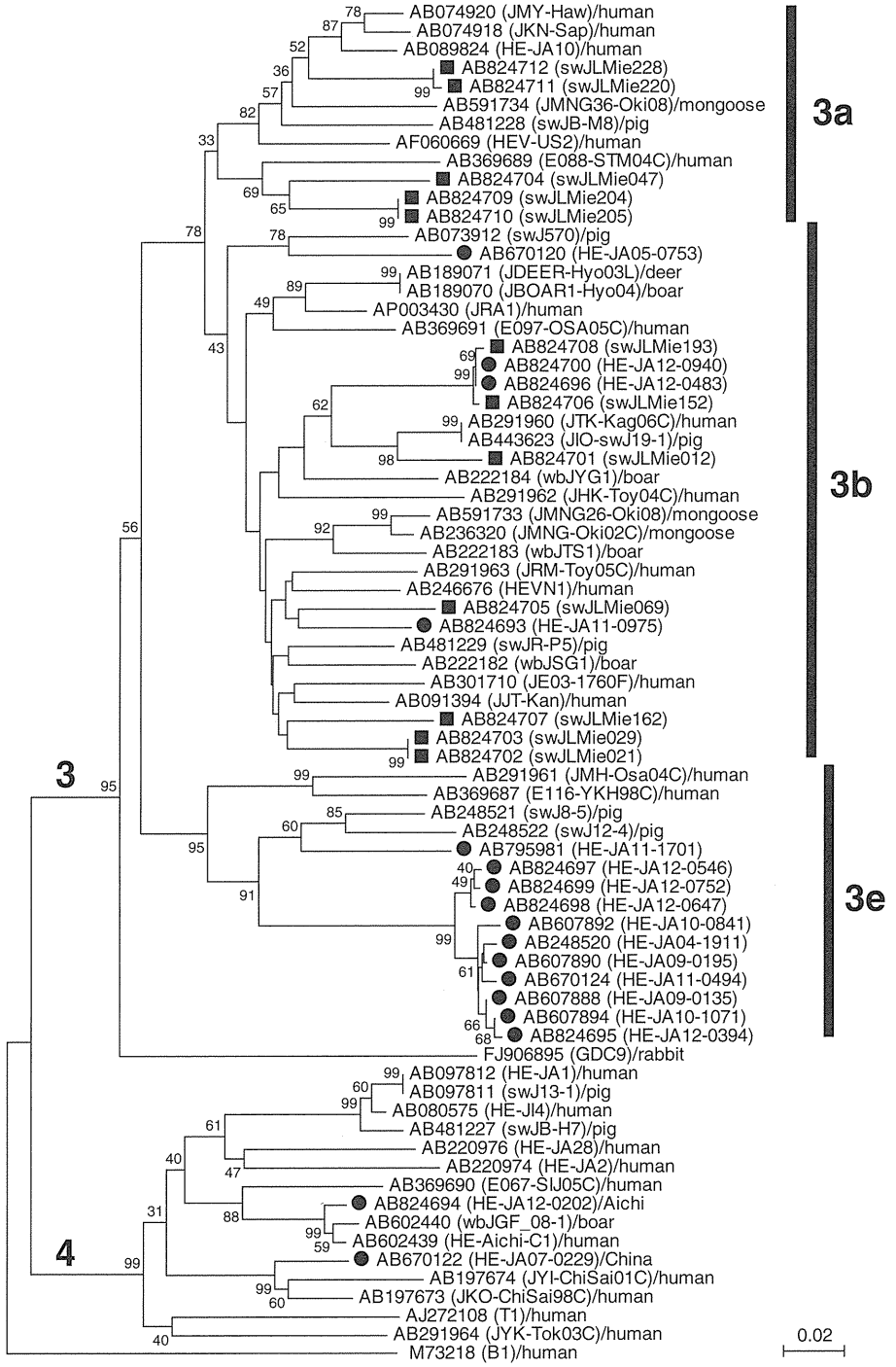

Figure 2 Neighbor-joining tree of the 412-nt open reading frame (ORF)2 sequences of the 17 human and 12 swine hepatitis E virus (HEV) strains obtained in this study, with 42 reference sequences of genotypes 3 and 4 and an outgroup isolate of genotype 1 (M73218). The reference sequences are shown with 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.

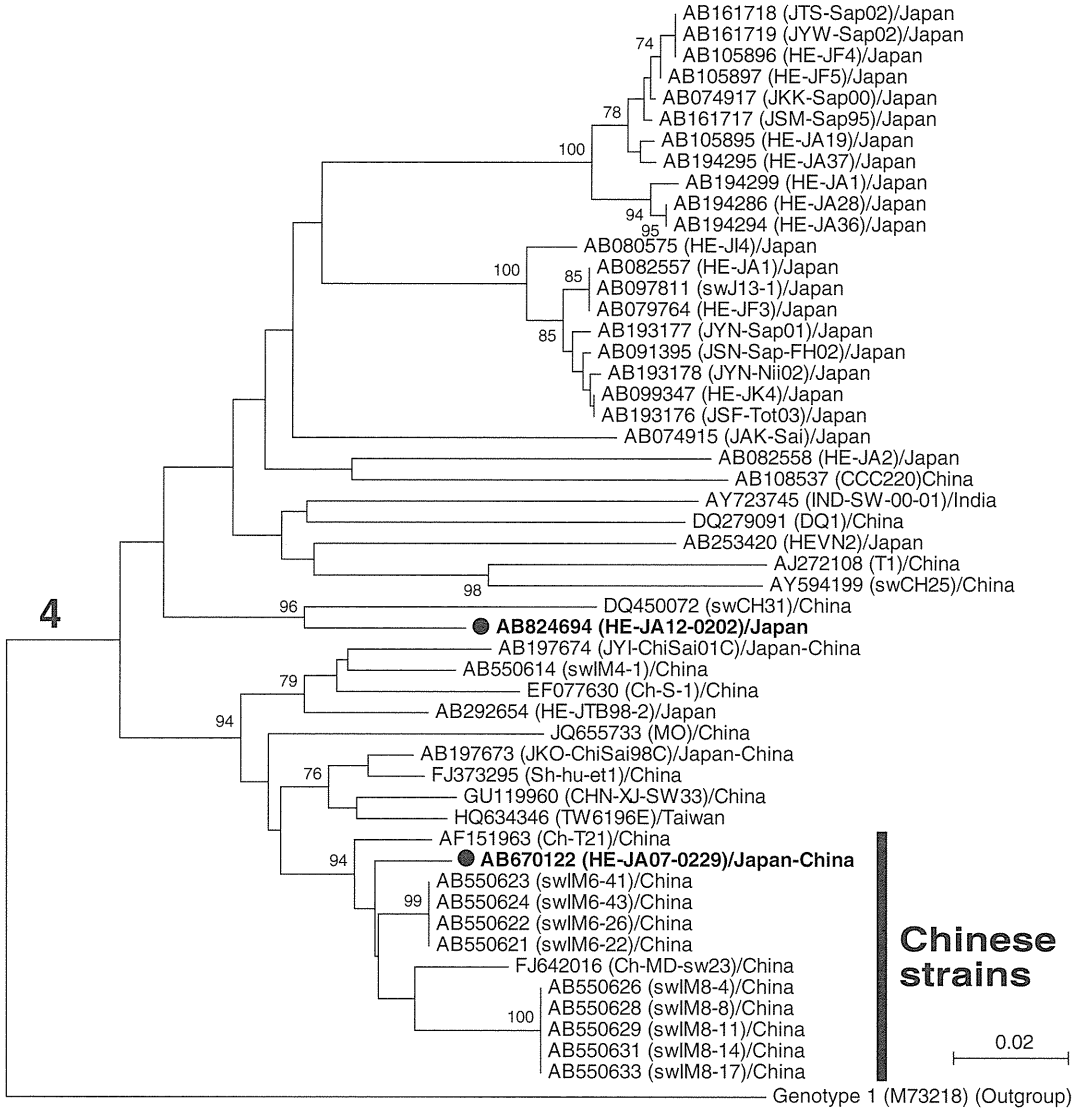

Figure 3 Neighbor-joining tree of the 412-nt reading frame (ORF)2 sequences of the genotype 4 hepatitis E virus (HEV) strains (HE-JA07-0229 and HE-JA12-0202) obtained in this study, with 49 reference sequences of genotype 4 and an outgroup isolate of genotype 1 (M73218). The reference sequences are shown 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

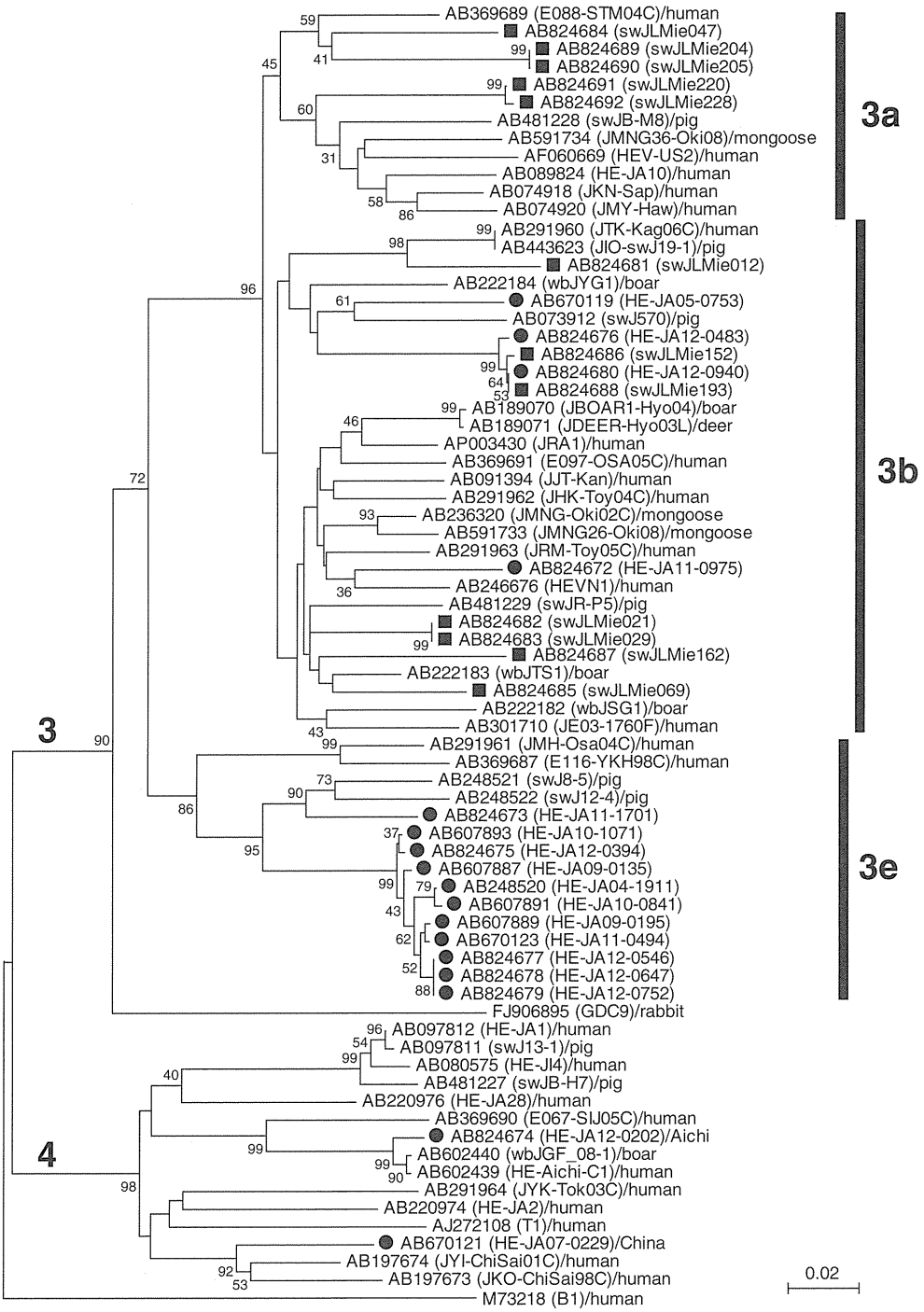

Figure 4 Neighbor-joining tree of the 412-nt open reading frame (ORF)1 sequences of the 17 human and 12 swine hepatitis E virus (HEV) strains obtained in this study, with 42 reference sequences of genotypes 3 and 4 and an outgroup isolate of genotype 1 (M73218). The reference sequences are shown with 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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Full Genome Analysis of a European-Type Genotype 3 Hepatitis E Virus Variant Obtained From a Japanese Patient With Autochthonous Acute Hepatitis E

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A unique European-type HEV strain (HE-JA12-0725) classifiable into subgenotype 3f was recovered from a 66-year-old Japanese female with autochthonous acute hepatitis E, and its entire genomic sequence was determined and characterized. The HE-JA12-0725 strain shared the highest identity of 92.7% with a Spanish swine isolate (EU723514) over the entire genome and possessed a long hypervariable region sequence of 111 amino acids, identical to the 3f strains of European origin. The patient had consumed pork liver obtained via home delivery in Japan approximately two months before the disease onset. These results suggest the circulation of rare 3f HEV strains in Japan. ***J. Med. Virol. 00: 1–5, 2015.***

KEY WORDS: hepatitis viruses; genotype; hypervariable region; phylogenetic tree; autoimmune hepatitis

Hepatitis E virus (HEV), a member of the genus *Hepevirus* in the family *Hepeviridae*, is the causative agent of hepatitis E. Accumulating evidence indicates that HEV can be transmitted zoonotically to humans, and that sporadic acute hepatitis E is able to develop via the ingestion of meat/viscera from domestic pigs or wild animals (e.g., boars and deer) [Tei et al., 2003; Yazaki et al., 2003; Li et al., 2005].

The HEV genome is a single-strand, positive-sense RNA of approximately 7.2 kilobases, and contains a short 5'-untranslated region (UTR), three open reading frames (ORFs: ORF1, ORF2, and ORF3) and a short 3'-UTR terminated by a poly(A) tract [Tam et al., 1991]. Currently, there are four recognized genotypes of HEV that infect humans [Okamoto, 2007]. Genotypes 1 and 2 of HEV have been identified exclusively in humans, while genotypes 3 and 4 of HEV have been isolated from not only humans, but also from pigs, wild boars, and deer, among other animals [Meng, 2013; Takahashi and Okamoto, 2014] and, therefore, can cause zoonosis and sporadic acute hepatitis in both industrialized and developing countries.

In Japan, autochthonous HEV strains are of genotype 3 or 4. Japan-indigenous genotype 3 HEV strains have been provisionally classified into three lineages, 3jp, 3us, and 3sp [Takahashi and Okamoto, 2014]: the 3jp, 3us, and 3sp lineages correspond to subgenotypes 3b, 3a, and 3e, respectively, as proposed by Lu et al. [2006]. In contrast, the subgenotype 3f HEV strain has been shown to be circulating actively between humans and wild boar/pig population in whole Europe [Adlhoch et al., 2009; Legrand-Abravanel et al., 2009; Peralta et al., 2009]. HEV strains homologous to the European 3f strains have also been recovered from humans and pigs in Thailand [Siripanyaphinyo et al., 2009; Suwannakarn et al., 2010; Keawcharoen et al., 2013]. Nucleotide

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sequence data of two 3f strains from patients with acute hepatitis E in Japan are available from the DDBJ/EMBL/GenBank databases. However, little is known about the characteristics of the 3f strains present in Japan.

A 66-year-old Japanese female was referred to a Municipal Hospital in Gunma prefecture, located 80 km northwest of Tokyo, Japan, with a clinical diagnosis of acute liver injury in May 2012. Her laboratory data on admission showed an elevation in the levels of serum liver enzymes (aspartate aminotransferase, 1,141 IU/L and alanine aminotransferase, 920 IU/L). In addition, the total bilirubin level of this patient was elevated (2.3 mg/dl), and the prothrombin time was decreased (69%) (Supplementary Table S1). She had no history of traveling abroad within the past year or a history of alcohol intake, transfusion of blood/blood derivatives or intravenous drug abuse. However, she had eaten a portion of pork liver obtained via home delivery from a food company in Japan approximately two months before the onset of acute hepatitis.

A liver biopsy performed on the next day of the hospitalization showed nonspecific hepatitis with interface hepatitis and fatty changes, suggesting autoimmune hepatitis (AIH) or alcoholic hepatitis. Based on internationally accepted criteria [Alvarez et al., 1999], she was initially diagnosed with a probable AIH. After admission, the prothrombin activity decreased gradually (Supplementary Table S2). Therefore, she was treated with methylprednisolone pulse therapy (1,000 mg for one day) on day 7, followed by oral prednisolone (started at 40 mg daily on day 10 and then tapered) to prevent the development of fulminant hepatitis, because the prothrombin activity had decreased to 39%. Her serum transaminase levels and prothrombin activity were improved promptly, although the serum transaminase levels had started to decrease before steroid therapy. After the administration of prednisolone, her serum was found to be positive for the IgM and IgA classes of HEV antibodies. The detection of serum HEV RNA led to a definitive diagnosis of acute hepatitis E (Supplementary Table S1). The HEV RNA became undetectable on day 40. A decrease in the titer of IgA class antibodies occurred, while the titer of IgG class antibodies increased with time (Supplementary Table S2). The patient recovered without any sequelae and was discharged on day 34.

The entire nucleotide sequence of the HE-JA12-0725 isolate recovered from the serum of the index patient was determined according to the previously described method [Okamoto et al., 2001]. The HE-JA12-0725 isolate had a genomic length of 7,317 nucleotides (nt), excluding the poly(A) tract at the 3′ terminus. This was the longest among all known HEV isolates whose entire sequence has been determined. The difference was attributed to an insertion of 120 nt in the hypervariable region (HVR) of ORF1 in this isolate, as compared with the prototype genotype 1 isolate (M73218), which has the HVR sequence of 213 nt. The entire genomic sequence of the HE-JA12-0725 has been deposited into the GenBank/EMBL/DDBJ databases under accession number AB850879.

Upon comparison with the prototype HEV genomes of genotypes 1–4, the HE-JA12-0725 genome shared nucleotide sequence identities of only 75.2% with genotype 1 HEV (M73218), 74.2% with genotype 2 HEV (M74506), and 68.0% with genotype 4 HEV (AJ272108), while it was closer (81.6% identity) to genotype 3 HEV (AF060669). Of note, HE-JA12-0725 was the most similar to subgenotype 3f strains (87.7–92.7%), but less similar (81.6–85.3%) to subgenotype 3a, 3b, and 3e strains among the known genotype 3 HEV strains.

A phylogenetic tree constructed according to the neighbor-joining method with the Kimura two-parameter model and 1,000 replicates of bootstrap resampling [Tamura et al., 2013], based on the full-length genomic sequences of genotype 1–4 HEV isolates, confirmed that HE-JA12-0725 belonged to genotype 3 and segregated into a cluster consisting of 3f HEV strains, supported by a bootstrap value of 100% (Supplementary Fig. S1). Among the 3f strains, HE-JA12-0725 clustered with three Spanish swine strains (EU723514-EU723516), supported by a bootstrap value of 100%, but not with the Thai and previously reported Japanese strains.

Upon comparison with the entire or partial nucleotide sequences of 85 reported 3f strains from nine countries (Table I), the HE-JA12-0725 strain obtained in the present study exhibited relatively low similarity ranging from 87.7% to 94.7%. When restricted to the entire genomic sequences, HE-JA12-0725 shared the highest nucleotide sequence identity of 92.7% with a swine HEV strain (EU723516) isolated in Spain, while it was only 90.8–91.4% identical to two strains (AB291961 and AB369687) obtained in Japan.

When compared within three ORFs at the nucleotide and amino acid levels, ORF2 and ORF3 of HE-JA12-0725 were identical in length to those of all reported 3f strains and to other genotype 3 strains, including subgenotypes 3a, 3b, and 3e strains (1,980 nt or 660 aa and 339 nt or 113 aa, respectively), while the length of ORF1 was variable, ranging from 5,112 nt (1,704 aa) to 5,199 nt (1,733 aa) (Supplementary Table S3). The HE-JA12-0725 isolate had the longest ORF1 of 5,199 nt (1,733 aa), which was identical to three Spanish swine HEV isolates (EU723514-EU723516), two French human HEV isolates (EU495148 and JN906974) and two French swine HEV isolates (JN906975-JN906976), but dissimilar to the two 3f strains (AB291961 and AB369687) obtained in Japan, which had the shorter ORF1 of 5,112 nt (1,704 aa).

The HE-JA12-0725 strain possessed a long HVR of 111 aa, identical to the 3f strains of Spanish and French origin (EU723514-EU723516, EU495148, and JN906974-JN6976), but dissimilar to those of Thai origin (82 aa) (Fig. 1). The two Japanese 3f strains (AB291961 and AB369687) deposited in the public

TABLE I. Comparison of the Overlapping Partial or Entire Genomic Sequences of the HE-JA12-0725 Isolate Obtained in the Present Study With the 85 Previously Reported 3f HEV Strains

Country	Isolation source	No. of isolates compared	Length of nucleotide sequence compared[a]	Accession no.	Nucleotide identity (%)
France	Human	2	7,261–7,315	EU495148, JN906974	91.3
	Human	9	302–345	EF113903, EF113905, EU116332, EU495191, EU495193, EU495201, EU495218, EU495219, EU495226	91.1–94.1
	Pig	3	7,249–7,315	JN906975-JN906976, JQ953666	90.5–91.3
Germany	Human	1	7,246	FJ956757	90.8
Greece	Human	1	371	AF110388	92.9
Netherlands	Pig	12	242, 304 or 476	AF332620, AF335998, AF336003, AF336005, AF336009, AF336010, AF336292, AF336294, AF336295, AF336296, AY932757, AY032759	90.7–93.4
Spain	Human	12	148–371	AF195061-AB195062, AF195064, AF195065, AF491000, AF491001, DQ141128, DQ141130, DQ383739, DQ383743, DQ383745, EF523421	90.6–94.0
	Pig	5	7,192–7,304	EU723512-EU723516	87.9–92.7
	Pig	6	168–304	AF195063, AY323506, DQ093568, DQ315745, DQ315763, DQ315770	90.6–94.6
Sweden	Pig	1	7,229	EU360977	87.7
New Caledonia	Pig	8	288–341	GU953683-GU953690	89.9–90.9
Thailand	Human	1	7,216	FJ653660	90.9
	Pig	1	7,245	EU375463	90.5
	Pig	20	415	EU708706-EU708725	91.3–93.0
	Wild boar	1	415	JN671918	90.8
Japan	Human	1	7,241	AB291961	90.8
	Human (with a history of travel to Thailand)	1	7,217	AB369687	91.4
Total		85	168–7,315		87.7–94.6

[a]The poly(A) sequence at the 3' terminus was excluded.

databases also had a shorter HVR of 82 aa, identical to the Thai 3f strains.

The present study revealed the presence of a new lineage of European-type genotype 3 (subgenotype 3f) HEV in a Japanese patient with autochthonous acute hepatitis E. Based on a phylogenetic analysis of the entire genomic sequences and a comparative analysis of the HVR sequences, the 3f strain (HE-JA12-0725) obtained in the present study was found to be similar to those circulating in Spain, but different from the two previously reported 3f strains recovered from hepatitis patients in Japan which were grouped into a cluster with Thai 3f strains, supported by a high bootstrap value (Supplementary Fig. S1) and the length of HVR sequence (Fig. 1). Since the HE-JA12-0725 strain was recovered from a Japanese patient who contracted autochthonous acute hepatitis E without a history of traveling abroad during the past year, it is likely that European-type 3f HEV strains are also rarely circulating in Japan.

The index patient in the present study consumed pig liver that may have been undercooked approximately two months before the onset of hepatitis, suggesting that it would be a strong candidate as the infectious source in this patient. The liver was purchased from a food company in Japan via home delivery. Unfortunately, the origin of the pig liver was unclear. However, since pig liver as food is generally made available at low cost, it is unlikely that the liver was imported from Europe or Thailand, thus suggesting that the liver containing the HE-JA12-0725 strain obtained in the present study had most likely originated from a pig raised in Japan, although the 3f HEV strains have never been identified in domestic pigs and wild animals (boars and deer) in Japan [Takahashi and Okamoto, 2014].

Of note, 3f HEV strains have thus far been isolated from only two HEV-infected subjects in Japan, both of which were made available only in the DNA databases. A Japanese isolate with accession no. AB291961 was isolated in Osaka in 2004. The other isolate, accession no. AB369687 was recovered from a patient with acute hepatitis E in Kanagawa in 1998 who had a history of travel to Thailand before onset. The subgenotype 3f strains have been considered to be of European origin. However, as mentioned above, the 3f strains have also been isolated in areas outside of Europe, including Thailand [Siripanyaphinyo et al., 2009; Suwannakarn et al., 2010; Keawcharoen et al., 2013]. Of interest, the two previously reported 3f strains in Japan were grouped into a cluster comprising Thai 3f strains, supported by a bootstrap value of 96% (Fig. 1), thus suggesting that they share common ancestor. In contrast, the HE-HE12-0725