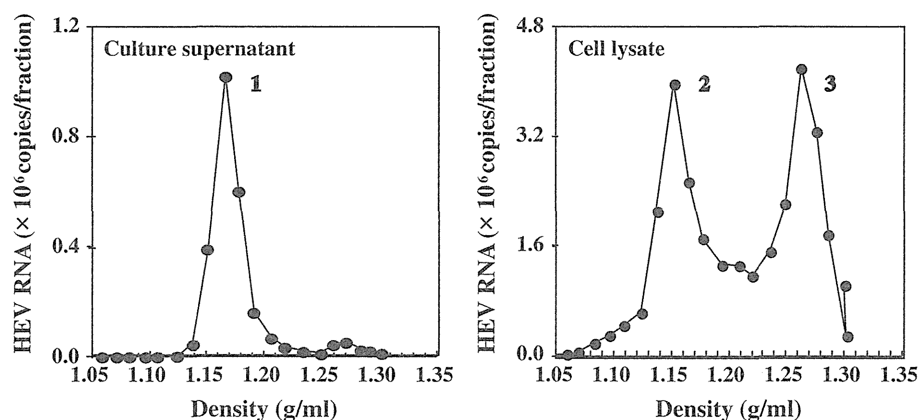

Fig. 6 Sucrose density gradient fractionation of the wild-type virus in the culture supernatants and cell lysates. The peak fractions (numbers 1 to 3) were subjected to immunocapture RT-PCR (see Table 3)

Table 3 Reactivity of TA1708, anti-ORF2 and anti-ORF3 mAbs with HEV particles in the culture supernatants and cell lysates with or without prior treatment with detergent, as evaluated using immunocapture RT-PCR

Virus[a]	% of captured HEV in the total HEV per well		
	mAb TA1708	mAb H6225 (anti-ORF2)	mAb TA0536 (anti-ORF3)
Without pre-treatment with detergent			
Culture supernatant			
Fraction 1 (1.16 g/ml)	46.3	9.5	9.1
Cell lysate			
Fraction 2 (1.15 g/ml)	46.0	11.6	9.6
Fraction 3 (1.26 g/ml)	0.7	64.5	2.0
With pre-treatment with detergent[b]			
Culture supernatant			
Fraction 1 (1.16 g/ml)	13.5	62.2	61.0
Cell lysate			
Fraction 2 (1.15 g/ml)	13.9	64.3	90.6
Fraction 3 (1.26 g/ml)	0.0	81.5	0.8

[a] Viruses were derived from the culture supernatant or lysate of pJE03-1760F/wt-transfected cells at 28 days post-transfection, and the peak fractions, with the indicated fraction number and sucrose density in parentheses (see Fig. 3), were subjected to immunocapture RT-PCR

[b] Prior to performing the immunocapture RT-PCR assay, 6 μl of the sucrose fraction was mixed with 60 μl of 0.11 % sodium deoxycholate, incubated at 37 °C for 2 hours, and then diluted 1:10 with PBS containing 0.1 % BSA

Discussion

Our previous study showed that an intact PSAP motif in the ORF3 protein is required for the formation and release of membrane-associated HEV particles possessing ORF3 proteins on the surface [30]. Moreover, we demonstrated that HEV recruits Tsg101 via its PSAP motif in the ORF3 protein and requires the L-domain function for virion release from infected cells, and also that the enzymatic activity of Vps4 is involved in virus release [31]. These results suggest that, although HEV is known to be a non-enveloped virus, it requires the MVB pathway for its release from infected cells. However, the origin of the membrane has not yet been clarified. In the present study, we generated and utilized a murine mAb against membrane-associated HEV particles, designated TA1708, and found that the mAb TA1708 binds specifically to a component of the membrane on the surface of HEV particles in an immunocapture RT-PCR assay (Table 1). In addition, digitonin treatment revealed that the membrane on the surface of viral particles shed into the culture supernatant is a lipid membrane (Fig. 1 and Table 2).

It remains unknown whether membrane-associated HEV particles are produced in the cytoplasm or at the cell surface [2]. Many enveloped viruses are known to complete their replication cycle by budding from the plasma membrane [10, 16, 18]. HIV, Ebola virus and other RNA viruses utilize the cellular ESCRT machinery to promote their escape from host cells by redirecting ESCRT complexes to the cell surface, where they appear to drive the budding and fission of the viral particles [10, 14, 27]. In our previous study, an immunofluorescence analysis revealed that the ORF3 protein is co-localized with CD63 and Tsg101 in the cytoplasm of HEV-infected cells [30, 31], thus suggesting that mature membrane-associated HEV particles are generated before their release from the surface of infected cells. In this study, we analyzed the subcellular localization of the antigen recognized by mAb TA1708 using immunofluorescence confocal microscopy. Specific signals were observed only in the cytoplasm, not on the plasma membrane (Fig. 2). Furthermore, the antigens were co-localized with TGOLN2, which has been established to be a TGN marker (Fig. 4). HEV particles with lipid membranes and the ORF3 protein on their surface were found abundantly in the lysates of cells infected with wild-type HEV (Fig. 6 and Table 3). Taken together, these findings indicate that HEV forms membrane-associated particles in the cytoplasm, likely by utilizing the cellular ESCRT machinery in the cytoplasm, not at the cell surface.

Lai et al. [22] reported that HCV egress requires the motility of early to late endosomes, which is microtubule-dependent. Additionally, they postulated that following the assembly of virus particles in juxtaposition to lipid droplets, the HCV particles are transported through early to late endosomes to the plasma membrane, where the membrane of late endosomes is fused with the plasma membrane to release virions into the extracellular milieu. Recently, Tamai et al. [41] reported that the Hrs-dependent exosomal pathway plays an important role in HCV release. In most herpesviruses, the final envelopment occurs at the Golgi, post-Golgi compartments, such as the TGN, or endosomes [8, 13]. Mori et al. [29] found that human herpesvirus 6 (HHV-6) buds at TGN-associated membranes, which contain CD63 and TGN46, and incorporates CD63 into virions. In addition, the virions are released together with internal vesicles (exosomes) through MVBs via the cellular exosomal pathway [29]. In the present study, we found, using double immunofluorescence staining, that mAb TA1708, which was raised against membrane-associated HEV particles, specifically recognizes TGOLN2, an intracellular antigen derived from the TGN, (Figs. 4 and 5). These results suggest that TGOLN2 derived from the TGN is a surface antigen of membrane-associated HEV particles. Contrary to our expectation, TGOLN2 recombinant protein was not detectable by western blot analysis, even under non-reducing or non-denaturing conditions, or immunoprecipitation using mAb TA1708 (data not shown), suggesting that the epitope of TGOLN2 that is present on the surface of virus particles, recognizable by mAb TA1708, is conformational and likely to be different from those expressed on the TGOLN2 recombinant protein. However, in an immunofluorescence assay, the Myc-tagged TGOLN2 recombinant protein was detectable by mAb TA1708, probably due to the maintenance of the three-dimensional structure of the recombinant protein expressed in cells. The co-localization of the antigen recognized by mAb TA1708 with the Myc-tagged TGOLN2 recombinant protein demonstrated by immunofluorescence assay using TA1708 and anti-Myc mAbs (Fig. 5) supports our notion that mAb TA1708 recognizes TGOLN2.

TGOLN2 is a protein encoded by the TGOLN2 gene in humans [34]. It has alternative names, including TGN38, TGN46, TGN48, TGN51 and TTGN2 [20, 25, 34], reflecting the presence of several isoforms of TGOLN2, which are produced by alternative splicing. This gene encodes a type I integral membrane protein that is localized to the TGN, a major sorting station for secretory and membrane proteins [34]. The encoded protein cycles between the TGN and early endosomes and may play a role in exocytic vesicle formation. Syntaxin 6 protein is also located at the TGN and mediates TGN vesicle trafficking events, likely via transport from the TGN to the endosome [4]. However, the present immunofluorescence studies (Fig. 4) suggested that mAb TA1708 is an antibody against a TGN protein that is recognizable by antibodies raised against TGN38, TGN46 and TGOLN2 rather than syntaxin 6.

Our previous study showed that the MVB pathway is important for the release of HEV particles and that ORF3 proteins are co-localized with CD63 in the cytoplasm [31]. In the present study, it was found that mAb TA1708 reacts with the intracellular antigen TGOLN2, according to the findings of double staining immunofluorescence analysis (Fig. 4). These results suggest that ORF3 proteins are transported on to the membrane of MVB by the early endosome via the vesicular transport system of TGN. It is likely that endosomal transport is involved in the release of HEV particles.

In conclusion, the present study revealed that membrane-associated HEV particles are present abundantly in the lysates of infected cells. In addition, double immunofluorescence staining showed that membrane-associated HEV particles retain the antigenicity of TGOLN2 derived from the TGN on the surface of the particles. These results indicate that the membrane of membrane-associated HEV particles is derived from the intracellular membrane, not from the cell surface. Further studies are required to elucidate whether the lipid membrane on the surface of HEV particles is derived not only from the TGN, but also from the Golgi apparatus or endosomes and whether mature virions are released via the exosomal secretion pathway, similar to what has been observed for other enveloped viruses, such as HCV and HHV-6 [22, 29, 41].

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ORIGINAL ARTICLE

The complete genomes of subgenotype IA hepatitis A virus strains from four different islands in Indonesia form a phylogenetic cluster

Mulyanto · I Dewa Nyoman Wibawa · Joseph Benedictus Suparyatmo · Rifai Amirudin · Hiroshi Ohnishi · Masaharu Takahashi · Tsutomu Nishizawa · Hiroaki Okamoto

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Abstract Despite the high endemicity of hepatitis A virus (HAV) in Indonesia, genetic information on those HAV strains is limited. Serum samples obtained from 76 individuals during outbreaks of hepatitis A in Jember (East Java) in 2006 and Tangerang (West Java) in 2007 and those from 82 patients with acute hepatitis in Solo (Central Java), Denpasar on Bali Island, Mataram on Lombok Island, and Makassar on Sulawesi Island in 2003 or 2007 were tested for the presence of HAV RNA by reverse transcription PCR with primers targeting the VP1-2B region (481 nucleotides, primer sequences at both ends excluded). Overall, 34 serum samples had detectable HAV RNA, including at least one viremic sample from each of the six regions. These 34 strains were 96.3-100 % identical to each other and formed a phylogenetic cluster within genotype IA. Six representative HAV isolates from each region shared 98.3-98.9 % identity over the entire genome and constituted a IA sublineage with a bootstrap value of 100 %, consisting of only Indonesian strains. HAV strains recovered from Japanese patients who were presumed to have contracted HAV infection while visiting Indonesia were closest to the Indonesian IA HAV strains obtained in the present study, with a high identity of 99.5-99.7 %, supporting the Indonesian origin of the imported strains. These results indicate that genetic analysis of HAV strains indigenous to HAV-endemic countries, including Indonesia, are useful for tracing infectious sources in imported cases of acute hepatitis A and for defining the epidemiological features of HAV infection in that country.

Introduction

Hepatitis A virus (HAV) is an important causative agent of acute hepatitis in humans worldwide, and is transmitted primarily through the fecal-oral route by the consumption of contaminated food and water or by person-to-person contact, but it is rarely transmitted parenterally or sexually [9, 18]. Infection with HAV is endemic in developing

Deceased: R. Amirudin.

The nucleotide sequences of HAV isolates reported herein have been assigned DDBJ/EMBL/GenBank accession nos. AB839692-AB839697 (complete genomes) and AB839698-AB839731 (481-nt VP1-2B sequences).

Mulyanto
Immunobiology Laboratory, Faculty of Medicine, University of Mataram, Mataram, Indonesia

Mulyanto
West Nusa Tenggara Hepatitis Laboratory, Mataram, Indonesia

I. D. N. Wibawa
Gastroentero-hepatology Division, Department of Internal Medicine, School of Medicine, Udayana University, Denpasar, Bali, Indonesia

I. D. N. Wibawa
Sanglah General Hospital, Denpasar, Bali, Indonesia

J. B. Suparyatmo
Department of Clinical Pathology, Moewardi Hospital, University of Sebelas Maret, Solo, Indonesia

R. Amirudin
Department of Internal Medicine, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

H. Ohnishi · M. Takahashi · T. Nishizawa · H. Okamoto (✉)
Division of Virology, Department of Infection and Immunity, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan
e-mail: hokamoto@jichi.ac.jp

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countries, and the majority of individuals in these countries are exposed to HAV during early 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 [12].

HAV is a member of the genus *Hepatovirus* within the family *Picornaviridae* [15] and contains a positive-sense, single-stranded RNA genome of approximately 7.5 kilobases (kb) in length, with a single long open reading frame (ORF). The ORF of 2227 amino acids (aa) is organized into three functional regions termed P1, P2 and P3. P1 encodes the capsid polypeptides VP1-VP4, whereas P2 and P3 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 [9]. Although HAV displays only a single serotype, HAV strains isolated from different parts of the world have been classified into six genotypes (I to 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 [17]. Genotype I is the most prevalent worldwide, and subgenotype IA is more common than IB. Currently, the complete or nearly complete nucleotide sequence is available for at least 50 human HAV isolates of subgenotypes IA, IB, IIA, IIB, IIIA and IIIB [3, 5, 7, 8, 17, 20, 27], but there is limited or no information about the isolates in many developing countries where HAV is endemic.

In Asian countries, there is considerable variety in the seroprevalence of HAV infection, with some continuing to have high rates and others making a transition to moderate or low rates [36]. In Indonesia, one of the largest archipelagos in the world, consisting of more than 17,000 islands, a study from 1978 to 1981 found a very high seroprevalence rate, reaching a level of 95 % by the age of 10 years [2]. In studies in the mid-1990s, some urban communities had a moderate seroprevalence rate, while rural areas continued to experience a high rate [6, 13, 37].

Despite the high endemicity of HAV in Indonesia, there are scarce data on the HAV strains circulating in this country: only partial sequences of HAV strains in Bali are available [37]. Virtually nothing is known about the relatedness of HAV isolates from this country and those from other parts of the world, especially other Asian countries. This knowledge is important for establishing evolutionary relationships and information about HAV transmission events. This study presents the first molecular characterization of full-length HAV genomes in six cities on four islands in Indonesia and demonstrates the usefulness of genome analysis of HAV strains for tracing infectious sources in patients with hepatitis A imported from Indonesia.

Materials and methods

Serum samples

Serum samples were obtained from 76 individuals during outbreaks of acute hepatitis A for the purpose of screening for ongoing HAV infections in Jember (n = 7) on East Java in 2006 and Tangerang (n = 69) on West Java in 2007 (Fig. 1). In addition, serum samples were obtained from 44 patients, including 19 patients in Solo on Java Island, 17 patients in Mataram on Lombok Island, and eight patients in Makassar on Sulawesi Island, who were clinically diagnosed to have acute hepatitis in 2007. Serum samples obtained from 38 patients in Denpasar on Bali Island who had developed sporadic acute hepatitis in 2003 were also analyzed: there were no overlaps in patients between the present study and the previous study [37]. Serum samples from hepatitis patients were obtained at the first visit, and all serum samples were stored first at −20 °C in Indonesia and then at −80 °C after having been sent to Jichi Medical University, Japan, until testing. Hepatitis patients exhibited an acute illness, presenting with clinical signs or symptoms such as jaundice, dark urine, general fatigue, anorexia, nausea, vomiting and fever, and had a serum alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) level that was at least two and half times the upper limit of the normal level. Autoimmune liver disease was not excluded by the routine test. This study was approved by the Ethics Committee of the hospital, and tested individuals gave informed consent.

Serum samples were tested for IgM antibodies against HAV (anti-HAV IgM) by a chemiluminescence immunoassay (Abbott Japan, Tokyo, Japan); hepatitis B surface antigen (HBsAg) by the hemagglutination method (Mycell, Institute of Immunology Co. Ltd., Tokyo, Japan); hepatitis B virus (HBV) DNA by nested polymerase chain reaction (PCR) with primers targeting the S gene region [28]; and the IgM class of anti-HBc antibody by ELISA according to the previously described method [26]. Antibodies to hepatitis C virus (HCV) were assayed by the hemagglutination method (Fujirebio, Tokyo, Japan), and serum samples with anti-HCV antibodies were assayed for HCV RNA by reverse transcription (RT)-PCR using primers derived from well-conserved areas of the 5' UTR of the HCV genome [23]. The IgG class of antibodies against hepatitis delta virus (HDV) was assayed using an in-house enzyme-linked immunoassay (ELISA) [10]. The IgM and IgA classes of antibodies against hepatitis E virus (HEV) were also assayed using an in-house ELISA [29].

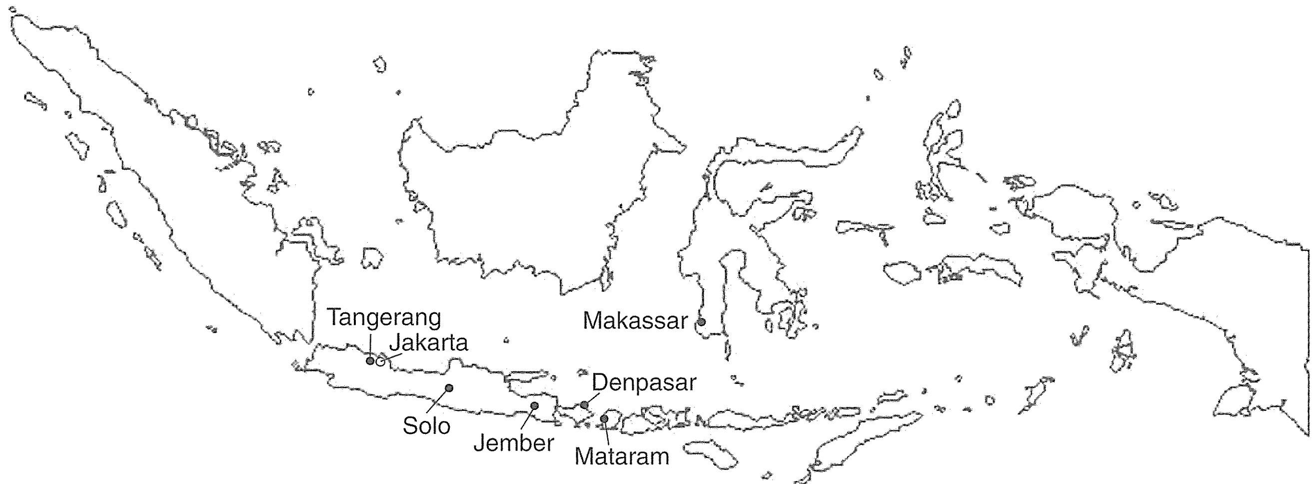


Fig. 1 Map of Indonesia, showing the locations of the six cities on four islands (Java, Bali, Lombok and Sulawesi) where serum samples were collected as well as the capital city, Jakarta

Detection of HAV RNA and determination of HAV genotypes

Serum samples were tested for the presence of HAV RNA by RT-PCR with nested primers derived from the VP1-2B region of the HAV genome as described previously [19]. Briefly, the RNAs extracted from 100 μl of serum were subjected to cDNA synthesis with reverse transcriptase (Superscript II; Life Technologies, Carlsbad, CA) and primer HA022 (5′-TTR TCA TCY TTC ATT TCT GTC C-3′ [R = A or G, Y = T or C]). The cDNAs were subjected to first-round PCR with TaKaRa Ex Taq (TaKaRa Bio, Shiga, Japan) and primers HA021 (5′-ATT GCA AAT TAY AAY CAY TCT GAT G-3′) and HA022 for 35 cycles (94 °C for 2 min before the start of cycling, then 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 60 s, with an additional 7 min for the last cycle). The second-round PCR was carried out for 25 cycles under the same conditions as the first-round PCR with primers HA023 (sense primer; 5′-CAT TCT GAT GAA TAY TTG TC-3′) and HA024 (antisense primer; 5′-CAT TTC TGT CCA TTT YTC ATC-3′). The amplification product of the first-round PCR was 548 base pairs (bp), and that of the second-round PCR was 522 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 522 bp was considered to be positive for HAV RNA. The HAV genotype was determined by a phylogenetic analysis of the amplified HAV sequence (481 nt: primer sequences at both ends excluded).

Amplification of full-length HAV genomes

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], TaKaRa LA Taq with GC Buffer [TaKaRa Bio] or TaKaRa Ex Taq) and primers whose sequences were derived from well-conserved areas across six subgenotypes (IA, IB, IIA, IIB, IIIA and IIIB) [8], 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 all six HAV isolates whose entire nucleotide sequences were determined in the present study. cDNAs covering the extreme 5′ end sequence (nt 1-61) were tailed with a dGTP homopolymer using a terminal deoxynucleotidyl transferase (New England BioLabs, Ipswich, MA) and amplified by single-sided PCR with 41-mer oligonucleotides containing $(C)_{15}$ according to a previously described method [22]. 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 [24].

Sequence analysis

The amplification products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co. Ltd., Tokyo, Japan), and both strands were then sequenced directly by employing an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). 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). Sequence analysis was performed using Genetyx software (version 11.1.2; Genetyx Corp., Tokyo, Japan), and multiple alignments were generated using CLUSTAL W, version 2.1 [32]. Phylogenetic trees were constructed by the neighbor-joining method [25], using the Kimura two-parameter correction model and 1,000 replicates of bootstrap re-sampling as implemented in the MEGA 5 software program (version 5.2.1: release #5130517) [30].

Fig. 2 Phylogenetic tree constructed by the neighbor-joining method ▶ using the genotype V isolate (D00924) as an outgroup, based on the partial nucleotide sequences of the VP1-2B region (481 nt). In addition to the 34 isolates obtained in the present study, 10 Balinese isolates as well as representative HAV isolates of genotypes/subgenotypes IA, IB, IIA, IIB, IIIA and IIIB whose overlapping 481-nt sequences were known were included for comparison. The HAV strains obtained in the present study are indicated by six different symbols representing the location where they were isolated in Indonesia (▲, Tangerang; Δ, Solo; ●, Jember; ○, Bali; ■, Mataram; □, Makassar). The previously reported HAV isolates are indicated with accession numbers followed by the names of isolates in parentheses and the names of the countries where they were isolated. Bootstrap values (≥70 %) are indicated for the major nodes as a percentage of the data obtained from 1,000 re-samplings. Bar: 0.02 nucleotide substitutions per site

Results

Prevalence of IgM anti-HAV and HAV RNA

All 158 serum samples were tested for the presence of anti-HAV IgM and HAV RNA, and 34 samples were found to be positive for HAV RNA, all of which also tested positive for anti-HAV IgM (Table 1): one sample from Bali was positive for anti-HAV IgM but negative for HAV RNA. In more detail, for the serum samples obtained during outbreaks of hepatitis A, five (7.2 %) of 69 samples in Tangerang and six (85.7 %) of seven samples in Jember had detectable HAV RNA, although none of the subjects had symptoms or signs of acute hepatitis. Among the serum samples obtained from patients with a clinical diagnosis of acute hepatitis, three (15.8 %) of 19 samples in Solo, 12 (31.6 %) of 38 samples in Denpasar, seven (41.2 %) of 17 samples in Mataram, and one (12.5 %) of eight samples in Makassar had detectable HAV RNA. A total of 34 viremic samples were subjected to HAV genotyping as described below.

Prevalence of other hepatitis virus markers

Although all 158 samples were negative for anti-HDV IgG and anti-HEV IgM/IgA, thus ruling out the possibility of acute HDV and HEV infections among the studied population, 11 samples were positive for anti-HBc IgM, accompanied by HBsAg and/or HBV DNA, leading to the diagnosis of acute hepatitis B (Table 1). Only one sample in Denpasar had anti-HCV, accompanied by HCV RNA,

Table 1 Demographic features and laboratory findings in patients with acute hepatitis or subjects whose serum samples were obtained for screening during outbreaks of hepatitis A in Indonesia[a]

Region (island/city)	No. of samples tested	Age (year, mean ± SD)	Male	ALT (IU/L, mean ± SD)	AST (IU/L, mean ± SD)	HAV RNA[b]	HBV markers		Anti-HCV
							HBsAg/HBV DNA[c]	Anti-HBc IgM	
Java									
Tangerang	69	20.3 ± 13.8	32 (46 %)	NT[d]	NT	5	0	0	0
Solo	19	42.4 ± 16.4	13 (68 %)	415.8 ± 480.9	432.0 ± 451.2	3	5	1	0
Jember	7	23.3 ± 11.3	4 (57 %)	NT	NT	6	0	0	0
Bali									
Denpasar	38	32.3 ± 11.9	32 (84 %)	904.0 ± 751.6	462.9 ± 376.3	12	16	8	1[e]
Lombok									
Mataram	17	29.7 ± 10.2	10 (59 %)	333.8 ± 124.5	300.4 ± 133.8	7	3	1	0
Sulawesi									
Makassar	8	35.5 ± 11.1	5 (63 %)	363.4 ± 141.4	213.5 ± 92.9	1	7	1	0
Total	158	27.7 ± 15.1	96 (61 %)	622.5 ± 622.0	397.3 ± 347.5	34	31	11	1

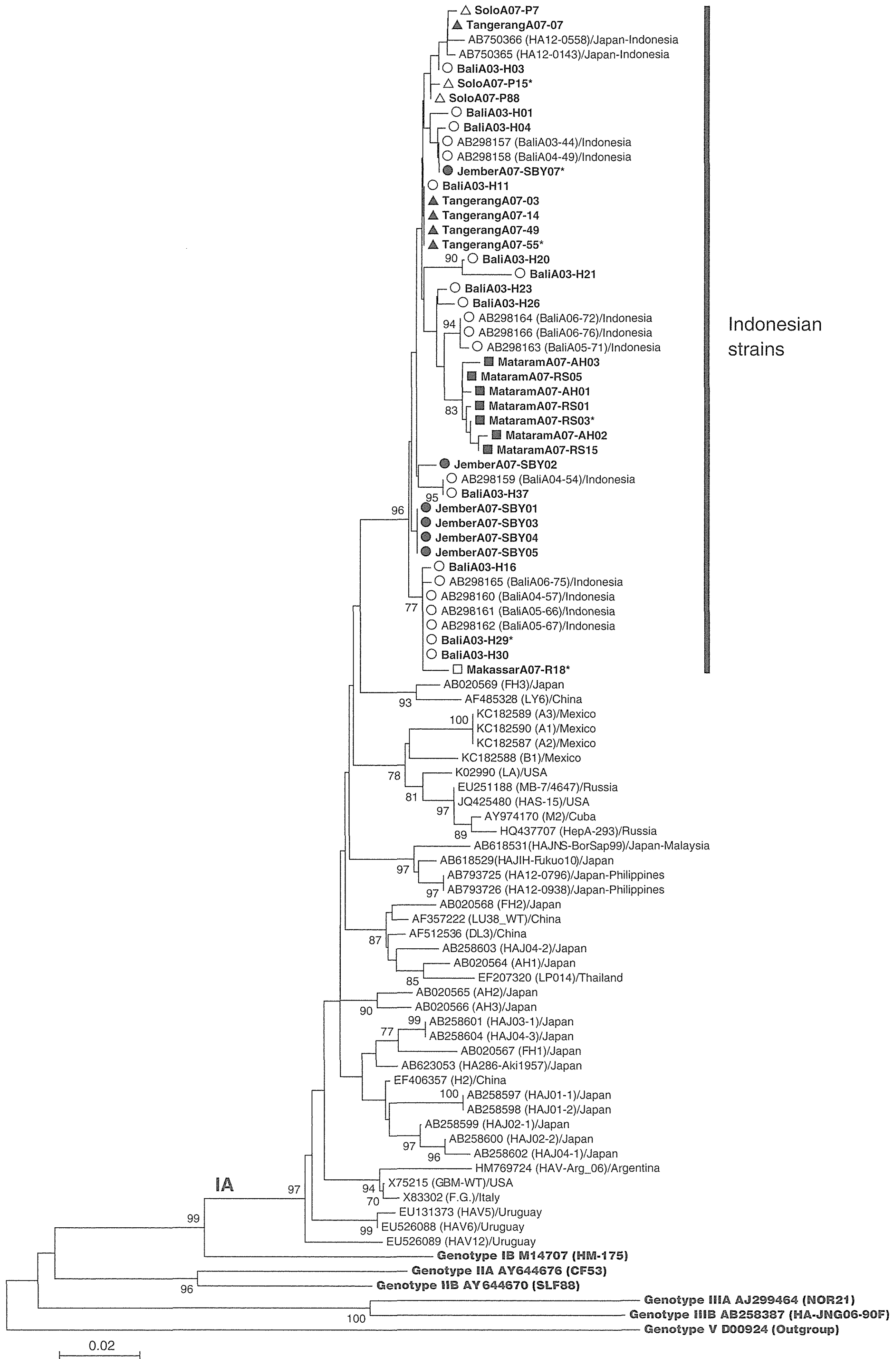
[a] All patients/subjects were negative for anti-HDV IgG and anti-HEV IgM/IgA

[b] All HAV RNA-positive samples were positive for IgM anti-HAV; one sample was positive for anti-HAV IgM but negative for HAV RNA in Bali

[c] Positive for HBsAg and/or HBV DNA, including 26 samples positive for both HBsAg and HBV DNA

[d] NT, not tested

[e] Positive for HCV RNA


SoloA07-P7
TangerangA07-07
AB750366 (HA12-0558)/Japan-Indonesia
AB750365 (HA12-0143)/Japan-Indonesia
BaliA03-H03
SoloA07-P15*
SoloA07-P88
BaliA03-H01
BaliA03-H04
AB298157 (BaliA03-44)/Indonesia
AB298158 (BaliA04-49)/Indonesia
JemberA07-SBY07*
BaliA03-H11
TangerangA07-03
TangerangA07-14
TangerangA07-49
TangerangA07-55*
BaliA03-H20
BaliA03-H21
BaliA03-H23
BaliA03-H26
AB298164 (BaliA06-72)/Indonesia
AB298166 (BaliA06-76)/Indonesia
AB298163 (BaliA05-71)/Indonesia
MataramA07-AH03
MataramA07-RS05
MataramA07-AH01
MataramA07-RS01
MataramA07-RS03*
MataramA07-AH02
MataramA07-RS15
JemberA07-SBY02
AB298159 (BaliA04-54)/Indonesia
BaliA03-H37
JemberA07-SBY01
JemberA07-SBY03
JemberA07-SBY04
JemberA07-SBY05
BaliA03-H16
AB298165 (BaliA06-75)/Indonesia
AB298160 (BaliA04-57)/Indonesia
AB298161 (BaliA05-66)/Indonesia
AB298162 (BaliA05-67)/Indonesia
BaliA03-H29*
BaliA03-H30
MakassarA07-R18*
Indonesian strains
AB020569 (FH3)/Japan
AF485328 (LY6)/China
KC182589 (A3)/Mexico
KC182590 (A1)/Mexico
KC182587 (A2)/Mexico
KC182588 (B1)/Mexico
K02990 (LA)/USA
EU251188 (MB-7/4647)/Russia
JQ425480 (HAS-15)/USA
AY974170 (M2)/Cuba
HQ437707 (HepA-293)/Russia
AB618531(HAJNS-BorSap99)/Japan-Malaysia
AB618529(HAJIH-Fukuo10)/Japan
AB793725 (HA12-0796)/Japan-Philippines
AB793726 (HA12-0938)/Japan-Philippines
AB020568 (FH2)/Japan
AF357222 (LU38_WT)/China
AF512536 (DL3)/China
AB258603 (HAJ04-2)/Japan
AB020564 (AH1)/Japan
EF207320 (LP014)/Thailand
AB020565 (AH2)/Japan
AB020566 (AH3)/Japan
AB258601 (HAJ03-1)/Japan
AB258604 (HAJ04-3)/Japan
AB020567 (FH1)/Japan
AB623053 (HA286-Aki1957)/Japan
EF406357 (H2)/China
AB258597 (HAJ01-1)/Japan
AB258598 (HAJ01-2)/Japan
AB258599 (HAJ02-1)/Japan
AB258600 (HAJ02-2)/Japan
AB258602 (HAJ04-1)/Japan
HM769724 (HAV-Arg_06)/Argentina
X75215 (GBM-WT)/USA
X83302 (F.G.)/Italy
EU131373 (HAV5)/Uruguay
EU526088 (HAV6)/Uruguay
EU526089 (HAV12)/Uruguay
IA
Genotype IB M14707 (HM-175)
Genotype IIA AY644676 (CF53)
Genotype IIB AY644670 (SLF88)
Genotype IIIA AJ299464 (NOR21)
Genotype IIIB AB258387 (HA-JNG06-90F)
Genotype V D00924 (Outgroup)
0.02

leading to a presumed diagnosis of acute hepatitis C. Even though patients with HBsAg and/or HBV DNA but without anti-HBc IgM are provisionally diagnosed with acute exacerbation of HBV infection, among the 82 patients with a clinical diagnosis of acute hepatitis, 29 patients (35.4 %) including 11 patients (57.9 %) in Solo, 10 patients (26.3 %) in Denpasar, seven patients (41.2 %) in Mataram, and one patient (12.5 %) in Makassar, had infections of unknown etiology (non-A to E).

Analysis of the VP1-2B region sequence of Indonesian HAV strains

A total of 34 HAV strains obtained in the present study were 96.3-100 % identical to each other within the 481-nt VP1-2B region sequence and were most closely related to HAV strains of subgenotype IA (n = 29), whose entire genomic sequence is known, with nucleotide identities of 94.1-96.8 %, compared to IB strains (88.3-90.8 % [n = 5]), IIA strains (82.7-84.5 % [n = 1]), IIB strains (84.3-85.6 % [n = 1]), IIIA strains (78.5-79.6 % [n = 15]), and IIIB strains (78.5-80.2 % [n = 4]). Of note, these 34 HAV strains shared high nucleotide sequence identity of 96.2-100 % with 10 previously reported Balinese strains [37]. A phylogenetic tree constructed based on the VP1-2B sequence confirmed that the 34 HAV strains obtained in the present study segregated into subgenotype IA and further formed a cluster with the reported Indonesian strains from Bali, supported by a high bootstrap value of 96 % (Fig. 2).

Characterization of the full-length Indonesian HAV genomes

The full-length genomic sequence was determined for a total of six Indonesian HAV strains selected from one each from 1 to 12 HAV strains in the six regions of Indonesia studied (TangerangA07-55, SoloA07-P15, JemberA07-SBY07, BaliA03-H29, MataramA07-RS03 and Makassar A07-R18). These six HAV strains 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 aa. 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 [4, 34].

The six HAV strains in Indonesia differed from each other by only 1.1-1.7 % over the entire genome, and they were 99.1-100 % identical to each other in the amino acid sequence of the polyprotein. Comparisons of the six HAV genomes against reported HAV genomes of genotypes I, II, III and V whose complete or nearly complete nucleotide sequences are known (see Fig. 3), confirmed that these six Indonesian HAV strains were most closely related to the human HAV isolates of subgenotype IA (n = 29), with identities of 94.5-97.2 % over the entire genome, and they were 90.5-91.4 % similar to the human HAV isolates of subgenotype IB (n = 5) and only 81.2-86.4 % 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 the six HAV strains segregated into subgenotype IA, with a bootstrap value of 100 %, and that they formed a cluster that was separate from the IA strains isolated in other countries, supported by a bootstrap value of 100 % (Fig. 3).

When the six Indonesian IA HAV strains whose full-length genomic sequences were determined in the present study were compared with 602 HAV strains of the same subgenotype from seven other Asian countries, including Malaysia, the Philippines, Taiwan, Thailand, Korea, China and Japan, whose overlapping coding region sequence of more than 200 nt was retrievable from the DDBJ/EMBL/GenBank databases as of July 2013, they shared only 91.1-98.7 % identity with the reported HAV strains from the seven Asian countries, except for two strains (HA12-0143 and HA12-0558) in Japan with 99.1 % identity, both of which were recovered from patients with imported hepatitis A who were presumed to have contracted the HAV infection while traveling in Indonesia [16].

Comparison of the two HAV strains that were presumed to have been imported from Indonesia with those in other Asian countries

Upon comparison of the HA12-0143 and HA12-0558 strains recovered from Japanese patients who were presumed to have contracted an HAV infection while visiting Indonesia [16] with those in eight Asian countries where subgenotype IA HAV sequences were available, it was found that these two strains were closest to those in Indonesia, especially in Tangerang, with the highest nucleotide sequence identity of 99.7 % and 99.5 %, respectively (Table 3). These two presumably Indonesia-indigenous strains shared nucleotide identities of only 93.1-98.8 % and 93.4-98.6 %, respectively, with those isolated from patients with domestic hepatitis A in Japan, thus suggesting that they were of non-Japanese origin.

Discussion

In the present study, we determined and analyzed six full-length and 28 partial (VP1-2B) genomic sequences of subgenotype IA HAV strains isolated in Indonesia. Of note, although these HAV strains were isolated in six cities on

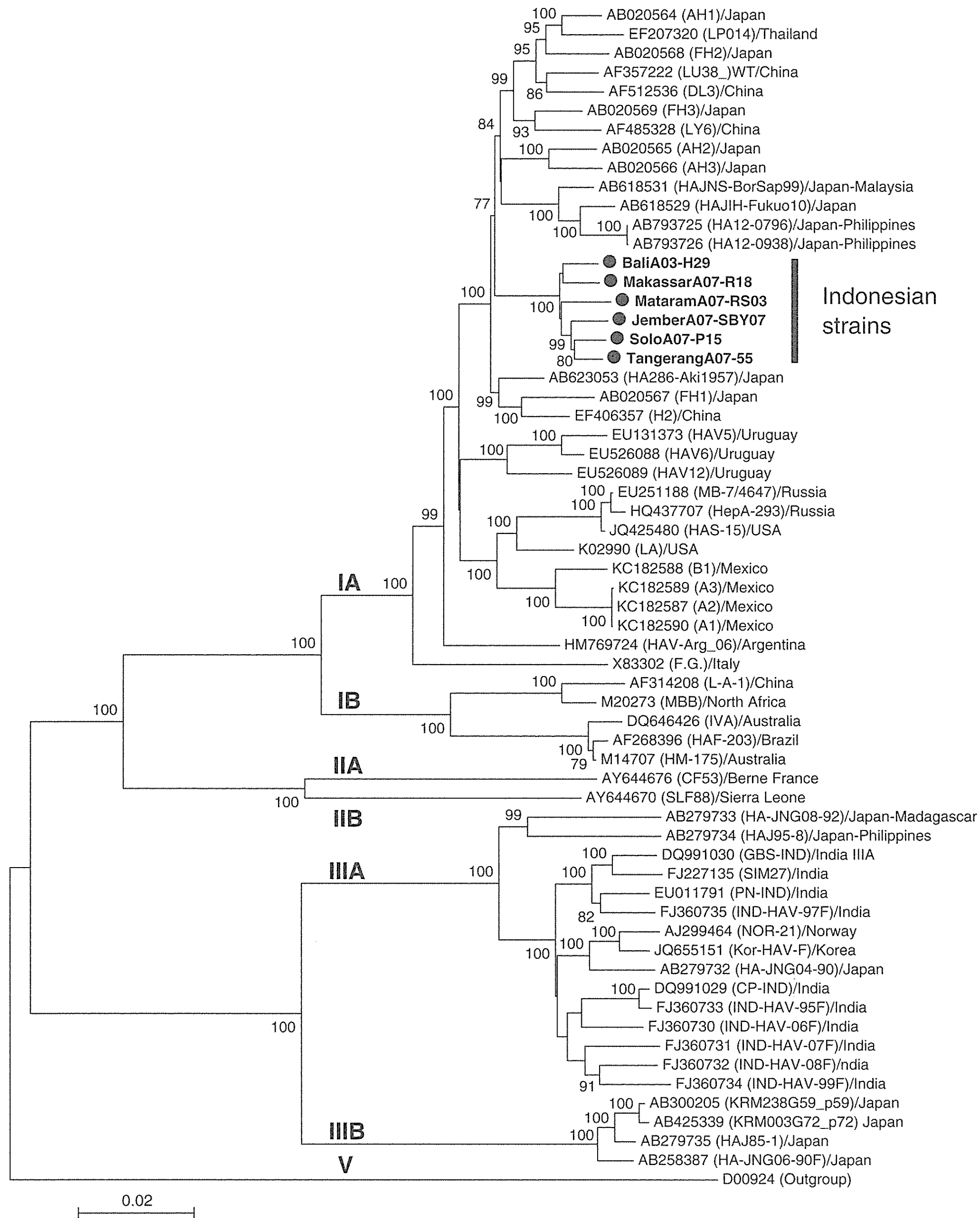


Fig. 3 Phylogenetic tree constructed based on the full-length nucleotide sequence of 52 HAV isolates by the neighbor-joining method using AGM-27 (D00924) as an outgroup. In addition to the six isolates of subgenotype IA obtained in the present study, which are indicated by the symbol "●" for visual clarity, 55 reported HAV isolates of genotypes/subgenotypes IA, IB, IIA, IIB, IIIA, IIIB and V whose complete or nearly complete sequences are known are included for comparison. The bootstrap values (≥70 %) are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings. Bar: 0.02 nucleotide substitutions per site

four different islands, which were approximately 1400 km apart from each other at the maximum distance, they formed a single phylogenetic cluster within subgenotype IA and constituted a IA sublineage composed of only Indonesian strains, clearly separate from those recovered from other Asian countries. Since no full-length genomic sequence data for HAV isolates in Indonesia have been determined thus far, the full-genome sequence data of the six HAV isolates in Tangerang (West Java), Solo (Central Java), Jember (East Java), Denpasar (Bali), Mataram (Lombok), and Makassar (Sulawesi) determined in the present study may be useful for molecular epidemiological studies on HAV infection as the prototype IA isolates circulating in this country.

Recently, the World Health Organization (WHO) published a systematic review on the global prevalence of HAV infection [36]. There is considerable variety in the seroprevalence rates in countries in Southeast Asia, with some continuing to have high rates and others shifting from high to moderate and moderate to low endemicity, with a

Table 2 Comparison of the six Indonesian subgenotype IA HAV isolates whose entire genomic sequences were determined in the present study with Asian HAV strains of the same subgenotype with a known coding region sequence of more than 200 nucleotides

HAV isolate	No. of isolates compared	Length of nucleotide sequence compared	Nucleotide sequence identity (%) to:					
			Tangerang A07-55	Solo A07-P15	Jember A07-SBY07	Bali A03-H29	Mataram A07-RS03	Makassar A07-R18
Malaysia	1	7493 nt	96.5	96.4	96.3	96.4	96.3	96.5
Philippines	14	228-7492 nt	95.3-98.7	95.3-98.7	95.3-98.7	94.5-97.8	92.8-96.4	93.5-97.3
Taiwan	73	855 nt	94.0-96.8	94.1-96.7	93.9-96.7	94.3-96.9	93.5-96.0	94.0-96.6
Thailand	11	200-7431 nt	95.9-97.2	95.9-97.2	95.9-97.2	95.6-96.9	94.0-95.9	94.7-96.1
Korea	77	232-900 nt	94.6-97.4	94.8-97.4	94.8-97.4	94.3-97.0	93.1-96.1	93.5-96.5
China	22	321-7487 nt	94.7-96.7	94.7-96.6	94.7-96.6	94.3-96.5	92.8-96.6	93.4-96.5
Japan	404	232-7494 nt	92.3-99.1	92.2-99.1	92.4-98.7	91.6-98.9	91.1-98.5	91.8-98.8
IA-1 (Ishii)[a]	11	232-475 nt	93.9-98.7	93.9-98.7	93.9-98.7	93.6-98.3	92.3-97.4	92.8-97.4
IA-2 (Ishii)[a]	30	232-7455 nt	95.4-96.2	95.5-96.2	95.5-96.2	95.1-96.0	93.5-95.9	94.0-96.0
Others	363	232-7494 nt	92.3-99.1	92.2-99.1	92.4-98.7	91.6-98.9	91.1-98.5	91.8-98.8

[a] Two IA sublineages (IA-1 and IA-2) described by Ishii et al. [11]

corresponding increase in the age of exposure from childhood to early adulthood. These changes have resulted from improvements in hygiene, sanitation and the quality of drinking water, reflecting improvements in the living standards and socioeconomic progress. In Indonesia, according to seroprevalence data from the mid-1990s, although some urban communities have a moderate rate of HAV seroprevalence, rural areas continue to have high seroprevalence [6, 13, 37]. The changes in HAV seroprevalence over time remain unclear, due to the absence of longitudinal studies assessing similar populations in Indonesia. Further studies using seroepidemiological and molecular virological approaches are clearly required to clarify the present status of HAV infection in this country in order to prevent HAV infection not only in residents but also in tourists from non-endemic countries, including Japan.

In Japan, the number of hepatitis A cases has been decreasing constantly since the 1990s due to improved sanitary conditions [19, 39]. However, the low incidence of infection resulted in increased susceptibility to HAV [14]. The growing susceptible population of advanced age leads to more frequent HAV infections among these individuals. Most of the infections that occurred in Japan were sporadic, with the rare occurrence of small-scale outbreaks such as those in revolving sushi bars or sushi shops [33]. Although the annual numbers of reported cases were low, at approximately 100-150 in the last several years, a total of 346 cases was reported in 2010 due to mini-epidemics in several regions of Japan, of which 61 cases were subjected to HAV genotyping, with IA (44 cases), IB (1 case) and IIIA (16 cases) all being detected [11]. The isolated IA strains were provisionally divided into two sub-lineages, including one (IA-1) corresponding to strains endemic to Japan and another (IA-2) having sequences that were very similar to each other and assumed to have been imported from one of the other Asian countries. The present study provided an opportunity to compare the IA-2 strains with Indonesia-indigenous HAV strains and clearly demonstrated that the IA-2 strains did not originate from Indonesia, based on the low nucleotide sequence similarity (≤96.2 %) with the Indonesian strains (Table 2).

Hepatitis A is one of the most common imported diseases in Japan [31]. Approximately 14 % of sporadic or cluster cases of hepatitis A during 2006-2008 in Japan were reported to have been imported from foreign countries such as India, the Philippines, Korea, Indonesia, China, Pakistan and Thailand [7, 11, 21, 35]. Recently, two Japanese patients (30- and 38-year-old males) with imported hepatitis A who were presumed to have contracted the disease while visiting Indonesia, based on their travel history and the relatively high nucleotide sequence identity of two HAV strains (HA12-0143 and HA12-0558: 98.7 % each) with the Balinese HAV strain (BaliA04-49 strain [AB298158]) were reported [16]. The present study revealed that the HAV strains recovered from these two patients were most closely related to the Tangerang strain (TangerangA07-07), with the highest nucleotide sequence identity of 99.7 % and 99.5 %, respectively (Table 3 and Fig. 2). Tangerang is a city in the Province of Banten, located approximately 25 km west of the capital city, Jakarta, which the two patients visited 3-5 weeks and 10 days, respectively, before the onset of disease, consistent with the known incubation period for hepatitis A, ranging from 10-50 days, with an average of approximately one month [9], strongly supporting the contraction of the HAV infection by these two Japanese patients while traveling in Jakarta. Based on the results obtained in the

Table 3 Comparison of the HA12-0143 and HA12-0558 isolates obtained from Japanese patients with hepatitis A imported from Indonesia with the Indonesian HAV strains obtained in the present study and Asian genotype IA HAV strains with an overlapping genomic sequence of more than 200 nucleotides

Country	No. of isolates compared	Length of nucleotide sequence compared	Nucleotide sequence identity (%)	
			HA12-0143	HA12-0558
Indonesia	44	481	96.8-**99.7**[a]	96.6-**99.5**[a]
Tangerang	5	481	99.1-**99.7**	98.9-**99.5**
Solo	3	481	99.1-99.5	98.9-99.3
Jember	6	481	98.5-98.7	98.5
Bali	22	481	96.8-99.5	96.6-99.3
Lombok	7	481	97.7-98.3	97.0-97.7
Makassar	1	481	98.1	97.9
Malaysia	1	481	95.2	95.0
Philippines	13	220-481	94.7-98.2	95.1-98.6
Thailand	10	200-358	95.5-96.9	95.3-96.6
Korea	52	232-427	94.6-97.8	94.3-97.4
China	22	316-481	90.2-96.6	90.4-96.4
Taiwan	83	240-481	94.3-98.7	94.6-98.5
Japan	250	218-481	93.1-98.8	93.4-98.6
IA-1 (Ishii)[b]	11	218-368	93.7-98.8	93.4-98.6
IA-2 (Ishii)[b]	30	223-481	95.4-96.5	95.1-96.9
Others	209	223-481	93.1-98.5	93.5-98.3

[a] The highest sequence identity is highlighted in bold

[b] Two IA sublineages (IA-1 and IA-2) described by Ishii et al. [11]

present study, we therefore suggest that the strategy for identifying the infection source in similar situations should be as follows: (i) for the accurate tracing of infectious sources of imported agents, entire genomic sequence should be determined for at least one representative (or predominant) HAV strain of each subgenotype in a country (preferably, for each sublineage within a subgenotype if polyphyletic strains belonging to multiple sublineages are circulating); (ii) if an entire genomic sequence of the possible source strain is available, one of the partial sequences such as VP1-2B (481 nt) and 3C/3D (590 nt) sequences, both of which have been demonstrated to be applicable to universal detection and the phylogenetic analysis of various strains [7], may thus be sufficient to trace the source of infection.

Surprisingly, 13-58 % of the patients studied had no serological or molecular markers of acute infection with HAV, HBV, HCV, HDV or HEV (collectively, non-A to E). The causative agent(s) of this hepatitis remain unknown. However, one possible cause is a third, previously unrecognized, enterically transmitted hepatitis agent, whose existence was suggested by Arankalle et al. [1], who could not find seroepidemiological evidence of HEV infection in one of 17 epidemics of non-A, non-B water-borne hepatitis in India. Recently, Xu et al. [38] reported the identification of a novel DNA virus, provisionally designated NIH-CQV, from acute or chronic non-A to E hepatitis patients in Chongqing, China. Unfortunately, however, NIH-CQV DNA was not detectable in any of the patients with disease of unknown etiology in the present study (data not shown). Given the transient occurrence of the acute markers of hepatitis viruses, the possibility that the higher percentage of "non-A to E" cases observed in the current study might be attributed in part to higher false negatives cannot be ruled out. In addition, in the present study, we did not exclude patients with a history of exposure to hepatotoxic drugs or chemicals and those with a past history of liver disease, so these etiologies might also have been involved.

In conclusion, the complete genomic sequences of the first Indonesia-indigenous subgenotype IA HAV isolates were determined in the present study. Although HAV strains were isolated from six different regions on four islands in Indonesia, they formed a single phylogenetic cluster, being classifiable into an Indonesian IA sublineage. The sequence data obtained in the present study may be useful for molecular epidemiological studies on HAV infection in Asian countries, especially in Indonesia and Japan, in relation to the clarification of source(s) of HAV infection or the chain of transmission through the exchange of people and food products between Japan and other countries. Since the seroepidemiological and molecular virological data on HAV infections in Indonesia are limited, and HAV strains circulating in other areas including Sumatra, Kalimantan, and Nusa Tenggara Islands and Papua Indonesia have not been studied, the surveillance of anti-HAV antibody prevalence and further analyses of HAV genomes implicated in acute hepatitis A are needed for implementing preventive measures and for controlling the spread of HAV in this country.

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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

Hiroshi Okano,[1]* Masaharu Takahashi,[8]* Yoshiaki Isono,[1] Hiroki Tanaka,[1] Tatsunori Nakano,[2] Yumi Oya,[3] Kazushi Sugimoto,[4] Keiichi Ito,[5] Shigeru Ohmori,[6] Tadashi Maegawa,[7] Makoto Kobayashi,[7] Shigeo Nagashima,[8] Tsutomu Nishizawa[8] and Hiroaki Okamoto[8]

[1]*Department of Gastroenterology, Suzuka General Hospital, Suzuka,* [2]*Department of Internal Medicine, Fujita Health University Nanakuri Sanatorium, Tsu,* [3]*Department of Gastroenterology, Mie Prefectural General Medical Center, Yokkaichi,* [4]*Department of Laboratory Medicine, Mie University School of Medicine, Tsu,* [5]*Department of Gastroenterology, Mie Prefectural Shima Hospital, Shima,* [6]*Department of Gastroenterology, Kuwana East Medical Center, Kuwana,* [7]*Department of Gastroenterology, Yokkaichi Municipal Hospital, Yokkaichi, and* [8]*Division of Virology, Department of Infection and Immunity, Jichi Medical University School of Medicine, Shimotsuke, Japan*

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.

Correspondence: Dr Hiroaki Okamoto, Division of Virology, Department of Infection and Immunity, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan. Email: hokamoto@jichi.ac.jp
*These authors contributed equally to this work.
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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.

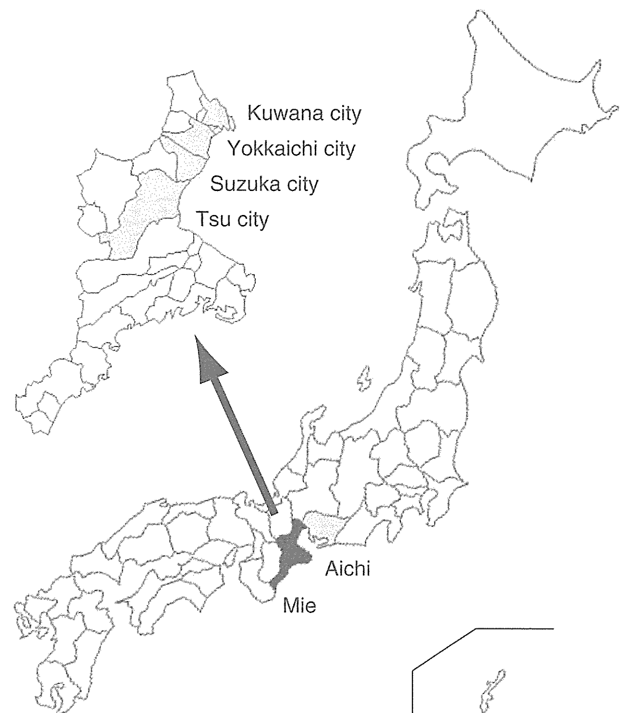

Figure 1 Map of Japan showing two prefectures (Mie and Aichi) and a magnified map of Mie prefecture showing the four cities where hepatitis E patients were identified in the present study. One (no. 11) of the 17 patients studied ingested meat from a wild boar in Aichi prefecture.

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

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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.

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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

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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.

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