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Unchanged high prevalence of antibodies to hepatitis E virus (HEV) and HEV RNA among blood donors with an elevated alanine aminotransferase level in Japan during 1991–2006*

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Summary

Hepatitis E is rare in Japan but is occurring more frequently than previously thought. To investigate whether de novo subclinical infection of hepatitis E virus (HEV) has recently increased in Japan, HEV RNA was assayed in serum samples obtained from 4019 Japanese voluntary blood donors with alanine aminotransferase (ALT) of ≥61 IU/l, who are likely to have ongoing HEV infection, during 1991–2006. The overall rates of IgG-class antibody to HEV (anti-HEV IgG), anti-HEV IgM/IgA and HEV RNA among 3185 donors in 2004–2006 were comparable with those among 594 donors in 1998 (5.3 vs. 5.2%, 0.2 vs. 0.5%, and 0.2 vs. 0.3%, respectively). Among blood donors with ALT ≥ 201 IU/l in three groups according to the year of blood collection (1991–1995 [$n=156$], 1996–1999 [$n=116$] and 2004–2006 [$n=61$]), there were no appreciable differences in the prevalence of anti-HEV IgG (5.8, 4.3, and 6.6%, respectively), anti-HEV IgM/IgA (1.9, 3.4, and 3.3%, respectively) and HEV RNA (1.3, 3.4, and 3.3%, respectively). The eleven HEV isolates obtained in the present study differed from each other by 1.7–22.8% in the ORF2 sequence and segregated into genotype 3 or 4. The occurrence rate of subclinical infection with divergent HEV strains has essentially remained unchanged during 1991–2006 in Japan.

Introduction

Hepatitis E, which is caused by hepatitis E virus (HEV), is found in many parts of the world. The disease is transmitted via the fecal-oral route through virus-contaminated water or food in developing countries where sanitation is suboptimal [36]. HEV infection is also endemic in industrialized countries, and IgG-class antibodies against HEV (anti-HEV IgG), most likely due to past subclinical HEV

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infection, have been detected in a significant proportion of healthy individuals in the United States, European countries and Japan [8, 16, 33, 36, 40, 51]; however, only a limited number of sporadic cases of acute hepatitis E have been reported in industrialized countries. Increasing lines of evidence indicate that hepatitis E is a zoonosis and that there exist animal reservoirs of HEV [9, 24, 25, 31–33, 40, 48, 52, 57].

HEV is a single-stranded, positive-sense RNA virus without an envelope and is currently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [4]. Its genome is approximately 7.2 kilobases (kb) in length and contains three open reading frames (ORFs: ORF1, ORF2, and ORF3) flanked by short untranslated regions [49]. ORF1 encodes non-structural proteins that are involved in virus replication and viral protein processing. ORF2 encodes the capsid protein and ORF3 encodes a small phosphorylated protein [14, 58]. Due to the extensive genomic diversity noted among HEV isolates, HEV sequences have been classified into four genotypes (genotypes 1–4) [39, 56]. Genotype 1 HEV was responsible for a number of waterborne epidemics of hepatitis E in Asia and Africa. Although HEV of genotype 2 has been detected less frequently, it was responsible for outbreaks in Mexico in 1986–1987 [54] and has been implicated in sporadic infections in Africa [3, 20]. On the other hand, genotypes 3 and 4 HEV cause sporadic cases of acute hepatitis but have not been found to be responsible for epidemics in humans; these infections seem to be zoonotic and both genotypes have been detected in pigs (genotype 3 worldwide, and genotype 4 in East Asia), which may constitute the major reservoir of HEV genotypes 3 and 4 [8, 19, 24, 25].

In Japan, multiple HEV strains of genotypes 3 and 4 have been recovered from patients with domestically acquired hepatitis E [12, 30, 42–45], and HEV has been recognized as an important causative agent of sporadic acute hepatitis of non-A, non-B, non-C etiology [30, 33]. A high prevalence of anti-HEV IgG has been reported [16, 27, 28, 50, 51], and HEV-viremic subjects have been identified among symptom-free blood donors with an elevated alanine aminotransferase (ALT) level [7]. However, it remains unknown whether or not subclinical HEV infection is increasing recently in Japan. Therefore, in an attempt to investigate the changing prevalence of de novo subclinical HEV infection in Japan, HEV RNA was assayed in serum samples obtained from Japanese voluntary blood donors with an elevated ALT level of $\geq$61 IU/l, who are likely to have ongoing HEV infection, during 1991–2006.

Materials and methods

Serum samples

Serum samples were collected from a total of 3185 voluntary blood donors (age: 32.5 ± 10.9 [mean ± standard deviation, SD] years; 2863 men and 322 women) with an elevated ALT level of 61–967 (range: 87.9 ± 41.8, mean ± SD) IU/l at the Japanese Red Cross Tochigi Blood Center, Japan, between April 2004 and December 2006. The Blood Center is located in Tochigi Prefecture, a prefecture in the northern part of mainland Honshu of Japan. Serum samples collected from 594 blood donors with an elevated ALT level of 61–2178 (100.4 ± 106.9) IU/l between February and November 1998 at the same blood center were also used in the present study. In addition, serum samples obtained from 240 blood donors with an elevated ALT level of $\geq$201 IU/l at the same blood center, from 1991–1997 and 1999, were used. Serum samples obtained from repeat donors during the study period were excluded; that is, each sample was obtained from a unique individual.

All 4019 serum samples were negative for hepatitis B surface antigen, and antibodies to hepatitis C virus (HCV) and human immunodeficiency virus (HIV) type 1. The 3198 samples obtained since 1999 were additionally negative for hepatitis B virus DNA, HCV RNA and HIV type 1 RNA by the nucleic acid amplification test using Roche's Multiplex reagent [26].

Detection of antibodies to HEV

To detect anti-HEV IgG, anti-HEV IgM and anti-HEV IgA, enzyme-linked immunosorbent assay (ELISA) was performed using purified recombinant ORF2 protein of HEV genotype 4 that had been expressed in the pupae of silkworm [30], as described previously [47]. In the ELISA assays for anti-HEV IgG, anti-HEV IgM and anti-HEV IgA, peroxidase-conjugated mouse monoclonal anti-human IgG antibody, peroxidase-conjugated mouse monoclonal anti-human IgM, or peroxidase-labeled mouse monoclonal anti-human IgA, respectively, was used. The optical density (OD) of each sample was read at 450 nm. The cut-off value used for the anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA assays was 0.175, 0.440, and 0.642, respectively [47]. Samples with OD values for anti-HEV IgG, IgM, or IgA equal to or greater

than the respective cut-off value were considered to be positive for anti-HEV IgG, IgM, or IgA, respectively. The specificity of the anti-HEV assays was verified by absorption with the same recombinant ORF2 protein that was used as the antigen probe. Briefly, when the OD value of the tested sample was less than 30% (anti-HEV IgG/IgA) or 50% (anti-HEV IgM) of the original value after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV.

Detection of HEV RNA

Reverse transcription (RT)-polymerase chain reaction (PCR) was performed for detection of HEV RNA in serum samples with anti-HEV IgM and/or anti-HEV IgA, using nested primers targeting the ORF2 region, as described previously [30]. The size of the amplification product of the first-round PCR was 506 base pairs (bp), and that of the second-round PCR was 457 bp. The nested RT-PCR assay that we used has the capability of amplifying all four known genotypes of HEV strains reported thus far [30, 46, 57]. The specificity of the RT-PCR assay was verified by sequence analysis as described below. The sensitivity of the RT-PCR assay was assessed as described previously [30]. For serum samples that were negative for HEV RNA when 100 µl of serum sample was used, total RNA was extracted from 500 µl of serum, reverse transcribed, and then subjected to nested PCR as described above. To extract RNA from 500 µl of serum, test serum diluted 2-fold in saline was centrifuged at 287,582 × *g* at 4 °C for 2 h in a TLA-100.2 rotor (Beckman Coulter K. K., Tokyo, Japan), and the resulting pellet was suspended in 100 µl of saline and subjected to the RT-PCR assay. To confirm the reproducibility, this assay was performed in duplicate.

For serum samples without anti-HEV IgM and anti-HEV IgA, 10 µl each from 50 serum samples were pooled, and each pool was tested for HEV RNA by the above-mentioned RT-PCR. If a pool was positive for HEV RNA, the 50 serum samples of that pool were individually tested for the presence of HEV RNA.

Sequence analysis of PCR products

The amplification products were sequenced directly on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Genetyx-Mac version 12.2.7 (Genetyx Corp., Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ: National Institute of Genetics, Mishima, Japan) [11]. Sequence alignments were generated by CLUSTAL W (version 1.8) [53]. Phylogenetic trees were constructed by the neighbor-joining method [38] based on the partial nucleotide sequence of the ORF2 region (412 nucleotides [nt]). Bootstrap values were determined on 1000 resamplings of the data sets [6].

Statistical analysis

Statistical analyses were performed using the Chi-Square-test for comparison of proportions between two groups. Differences were considered to be statistically significant at $P<0.05$.

Results

Age- and sex-specific prevalence of anti-HEV antibodies and HEV RNA during 2004–2006

A total of 3185 serum samples obtained from apparently healthy blood donors with an elevated ALT level between April 2004 and December 2006 were tested for the presence of anti-HEV IgG. Anti-HEV IgG was detected in 5.3% (168/3185) of the tested population including 5.1% of the 2863 male donors and 6.5% of the 322 female donors, the difference not being significant (Table 1). The prevalence of anti-HEV IgG increased with age among both the male and female donors, and was significantly higher among donors aged ≥40 years than among those aged <40 years in total (10.9 vs. 3.4%, $P<0.0001$) and in the males (11.0 vs. 3.2%, $P<0.0001$). All 168 serum samples with anti-HEV IgG were tested for anti-HEV IgM and anti-HEV IgA. Among them, anti-HEV IgM and anti-HEV IgA were simultaneously detected in six samples (3.6%), of which four samples tested positive for HEV RNA in a sample volume of both 10 and 100 µl, and one sample in 500 µl. As for the 3179 serum samples without anti-HEV IgM and anti-HEV IgA, although sixty-three 50-sample pools and one 29-sample pool were tested for the presence of HEV RNA, none of them had detectable HEV RNA. Consequently, 5 (0.2%) of the 3185 samples were found to be viremic for HEV. As for the prevalence of HEV viremia, there were no appreciable differences between males and females (0.1 vs. 0.3%, $P=0.4628$), and between donors aged ≥40 years and those aged <40 years (0.4 vs. 0.1%, $P=0.0700$).

Prevalence of anti-HEV antibodies and HEV RNA during 2004–2006, stratified by ALT level

In the present study, 168 donors with anti-HEV IgG were found during 2004–2006, including 143 (5.6%) with an ALT level of 61–100 IU/l, 21 (3.6%)

Table 1. Age- and sex-dependent prevalence of anti-HEV antibodies and HEV RNA among voluntary blood donors with an elevated ALT level between April 2004 and December 2006

Age (years)	No. of total donors (%) with				No. of male donors (%) with				No. of female donors (%) with			
	No. of donors	Anti-HEV		HEV RNA	No. of donors	Anti-HEV		HEV RNA	No. of donors	Anti-HEV		HEV RNA
		IgG-class	IgM- and/or IgA-class			IgG-class	IgM- and/or IgA-class			IgG-class	IgM- and/or IgA-class	
16–19	538	9 (1.7)	1 (0.2)	1 (0.2)	462	7 (1.5)	1 (0.2)	1 (0.2)	76	2 (2.6)	0	0
20–29	702	19 (2.7)	1 (0.1)	1 (0.1)	640	14 (2.2)	1 (0.2)	1 (0.2)	62	5 (8.1)	0	0
30–39	1150	53 (4.6)	0	0	1054	48 (4.6)	0	0	96	5 (5.2)	0	0
40–49	561	55 (9.8)	2 (0.4)	1 (0.2)	518	54 (10.4)	2 (0.4)	1 (0.2)	43	1 (2.3)	0	0
50–59	200	24 (12.0)	0	0	169	19 (11.2)	0	0	31	5 (16.1)	0	0
60–68	34	8 (23.5)	2 (5.9)	2 (5.9)	20	5 (25.0)	1 (5.0)	1 (5.0)	14	3 (21.4)	1 (7.1)	1 (7.1)
Total	3185	168 (5.3)	6 (0.2)	5 (0.2)	2863	147 (5.1)	5 (0.2)	4 (0.1)	322	21 (6.5)	1 (0.3)	1 (0.3)

with an ALT level of 101–200 IU/l, and 4 (6.6%) with an ALT level of ≥201 IU/l (Table 2). The prevalence of anti-HEV IgG was comparable between donors with an ALT level of ≥201 IU/l and those with an ALT level of 61–200 IU/l (6.6 vs. 5.2%). As for the prevalence of HEV RNA, however, there was a significant difference between donors with an ALT level of ≥201 IU/l and those with an ALT level of 61–200 IU/l in total (3.3 vs. 0.1%, $P<0.0001$), in males (3.7 vs. 0.1%, $P<0.0001$), but not in females, probably due to the small number of female donors tested (0 vs. 0.3%, $P=0.8813$).

Comparison of the prevalence of anti-HEV antibodies and HEV RNA between donors with an elevated ALT level in 1998 and those in 2004–2006

The overall rates of anti-HEV IgG, anti-HEV IgM/IgA and HEV RNA among donors in 1998 were comparable with those among donors in 2004–2006 (5.2 vs. 5.3%, 0.5 vs. 0.2%, and 0.3 vs. 0.2%, respectively) (Table 3). The prevalence of anti-HEV IgG increased with age in the two year groups, although none of the two donors in the age group of 60–68 years in 1998 had anti-HEV IgG. As in the year group of 2004–2006, the prevalence of HEV RNA was significantly higher among donors with an ALT level of ≥201 IU/l than among those with an ALT level of 61–200 IU/l in 1998 (6.3 vs. 0%, $P<0.0001$).

Prevalence of anti-HEV antibodies and HEV RNA among donors with an elevated ALT level of ≥201 IU/l, stratified by the year group of blood collection

Table 4 compares various features of the blood donors with an elevated ALT level of ≥201 IU/l, who are likely to have ongoing HEV infection, in the three year groups (1991–1995, 1996–1999 and 2004–2006) according to the year of blood collection. There were no appreciable differences in the age distribution, gender ratio, ALT level and prevalence of anti-HEV IgG among the three year groups of 1991–1995, 1996–1999 and 2004–2006. The prevalence of anti-HEV IgM/IgA and HEV RNA, indicative of present HEV infection, was low at 1.9

Table 2. Prevalence of anti-HEV IgG and HEV RNA among voluntary blood donors with an elevated ALT level during 2004–2006, stratified by ALT level

ALT (IU/l)	Total			Male			Female		
	N	Anti-HEV IgG	HEV RNA	N	Anti-HEV IgG	HEV RNA	N	Anti-HEV IgG	HEV RNA
61–100	2546	143 (5.6%)	2 (0.1%)	2296	128 (5.6%)	1 (0.04%)	250	15 (6.0%)	1 (0.4%)
101–200	578	21 (3.6%)	1 (0.2%)	513	15 (2.9%)	1 (0.2%)	65	6 (9.2%)	0
201–967	61	4 (6.6%)	2 (3.3%)	54	4 (7.4%)	2 (3.7%)	7	0	0
Total	3185	168 (5.3%)	5 (0.2%)	2863	147 (5.1%)	4 (0.1%)	322	21 (6.5%)	1 (0.3%)

Table 3. Comparison of the prevalence of anti-HEV antibodies and HEV RNA among blood donors with an elevated ALT level between 1998 and 2004–2006

Feature	1998				2004–2006			
	N	Anti-HEV-positive (%)		HEV RNA-positive (%)	N	Anti-HEV-positive (%)		HEV RNA-positive (%)
		IgG-class	IgM- and/or IgA-class			IgG-class	IgM- and/or IgA-class	
Age (years)								
16–19	43	0	0	0	538	9 (1.7)	1 (0.2)	1 (0.2)
20–29	216	6 (2.8)	1 (0.5)	1 (0.5)	702	19 (2.7)	1 (0.1)	1 (0.1)
30–39	200	11 (5.5)	0	0	1150	53 (4.6)	0	0
40–49	108	10 (9.3)	1 (0.9)	0	561	55 (9.8)	2 (0.4)	1 (0.2)
50–59	25	4 (16.0)	1 (4.0)	1 (4.0)	200	24 (12.0)	0	0
60–68	2	0	0	0	34	8 (23.5)	2 (5.9)	2 (5.9)
ALT (IU/l)								
61–100	454	23 (5.1)	1 (0.2)	0	2546	143 (5.6)	3 (0.1)	2 (0.1)
101–200	108	7 (6.5)	0	0	578	21 (3.6)	1 (0.2)	1 (0.2)
201–2178	32	1 (3.1)	2 (6.3)	2 (6.3)	61	4 (6.6)	2 (3.3)	2 (3.3)
Total	594	31 (5.2)	3 (0.5)	2 (0.3)	3185	168 (5.3)	6 (0.2)	5 (0.2)

Table 4. Prevalence of anti-HEV antibodies and HEV RNA among blood donors with an elevated ALT level of ≥201 IU/l, stratified by the year group of blood collection

Feature	Year of blood collection		
	1991–1995 ($n=156$)	1996–1999 ($n=116$)	2004–2006 ($n=61$)
Age (mean ± SD, years)	26.0 ± 9.9	26.4 ± 9.9	27.6 ± 12.5
Male	145 (92.9%)	105 (90.5%)	54 (88.5%)
ALT (mean ± SD, IU/l)	294.6 ± 216.4	299.8 ± 211.6	289.4 ± 135.8
Anti-HEV IgG	9 (5.8%)	5 (4.3%)	4 (6.6%)
Anti-HEV IgM/IgA	3 (1.9%)	4 (3.4%)	2 (3.3%)
HEV RNA	2 (1.3%)[a,b]	4 (3.4%)[a,c]	2 (3.3%)[b,c]
HEV genotype			
Genotype 3	1 (50.0%)	2 (50.0%)	2 (100%)
Genotype 4	1 (50.0%)	2 (50.0%)	0

[a] $P=0.2290$.
[b] $P=0.3256$.
[c] $P=0.9528$.

and 1.3%, respectively, in the year group of 1991–1995, but the difference among the three year groups was not statistically significant.

Genetic analysis of HEV isolates recovered from 11 viremic donors

The 11 HEV isolates recovered from the transiently viremic donors were named with the prefix of HE-JTB followed by the year of isolation and the sequential number of the viremic samples obtained that year (Table 5). The 412-nt sequence of ORF2 of these HEV isolates was determined and compared with each other and with that of known HEV isolates of genotypes 1–4. These 11 HEV isolates were markedly variable, sharing nucleotide identities ranging from 77.2 to 98.3%, and were classifiable into two groups differing by 18.9–22.8%. Eight HEV isolates (HE-JTB95-1, HE-JTB96-1, HE-JTB96-2, HE-JTB05-1, HE-JTB05-2, HE-JTB06-1, HE-JTB06-2, and HE-JTB06-3) comprised one group and were close to the prototype Japanese isolate of genotype 3 (JRA1 [accession no. AP003430]) with nucleotide identities of 85.9–93.0%, and were only 77.9–80.3, 74.8–77.4, and 77.9–80.1% similar to the B1 isolate (M73218) of genotype 1, MEX-14 isolate (M74506) of genotype 2, and T1 isolate (AJ272108) of genotype 4, respectively. This finding suggests that these 8 HEV isolates are classifiable into genotype 3. The phylogenetic tree constructed based on the common 412-nt sequence within the ORF2 sequence confirmed that these 8 HEV isolates obtained in the present study belonged to genotype 3 and showed that they segregated into clusters consisting of Japanese HEV strains of the same genotype that had been recovered from hu-

Table 5. Characteristics of blood donors with an elevated ALT level who had detectable HEV RNA

Year of isolation	Age (years)/sex	ALT (IU/l)	Anti-HEV (OD_{450} value)			HEV RNA (μl)[a]			HEV genotype	Isolate name
			IgG-class	IgM-class	IgA-class	10	100	500		
1994	44/M	457	>3.000 (+)	2.325 (+)	2.137 (+)	+[b]	+	NT[c]	4	HE-JTB94-1
1995	58/M	2598	0.415 (+)	0.611 (+)	1.171 (+)	+	+	NT	3	HE-JTB95-1
1996	47/F	215	1.629 (+)	1.900 (+)	>3.000 (+)	+	+	NT	3	HE-JTB96-1
1996	49/M	262	1.624 (+)	0.981 (+)	1.983 (+)	+	+	NT	3	HE-JTB96-2
1998	29/M	628	0.127 (−)	1.272 (+)	0.146 (−)	+	+	NT	4	HE-JTB98-1
1998	54/M	2178	0.439 (+)	1.030 (+)	1.265 (+)	+	+	NT	4	HE-JTB98-2
2005	61/M	967	1.762 (+)	1.967 (+)	2.825 (+)	+	+	NT	3	HE-JTB05-1
2005	25/M	85	>3.000 (+)	2.792 (+)	2.683 (+)	+	+	NT	3	HE-JTB05-2
2006	60/F	63	1.170 (+)	0.665 (+)	2.928 (+)	+	+	NT	3	HE-JTB06-1
2006	17/M	138	2.278 (+)	1.804 (+)	2.939 (+)	−	−	+	3	HE-JTB06-2
2006	49/M	758	2.866 (+)	>3.000 (+)	2.313 (+)	+	+	NT	3	HE-JTB06-3

[a] HEV RNA was assayed using the indicated volume of serum samples.
[b] +, positive for HEV RNA; −, negative for HEV RNA.
[c] NT, not tested.

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2 region (412 nt) of 99 HEV isolates, using a genotype 1 HEV (M73218) as an outgroup. In addition to the HE-JTB95-1, HE-JTB96-1, HE-JTB96-2, HE-JTB05-1, HE-JTB05-2, HE-JTB06-1, HE-JTB06-2, and HE-JTB06-3 isolates found in the present study, which are indicated in bold type for visual clarity, 90 reported HEV isolates of genotype 3, whose common 412-nt sequence is known, are included for comparison. The reported isolates are indicated with the accession no. followed by the name of the country of isolation (non-Japanese origin only). An asterisk denotes human or swine HEV strains that were isolated in the same prefecture as those obtained in the present study. Bootstrap values are indicated for the major nodes as a percentage obtained from 1000 resamplings of the data

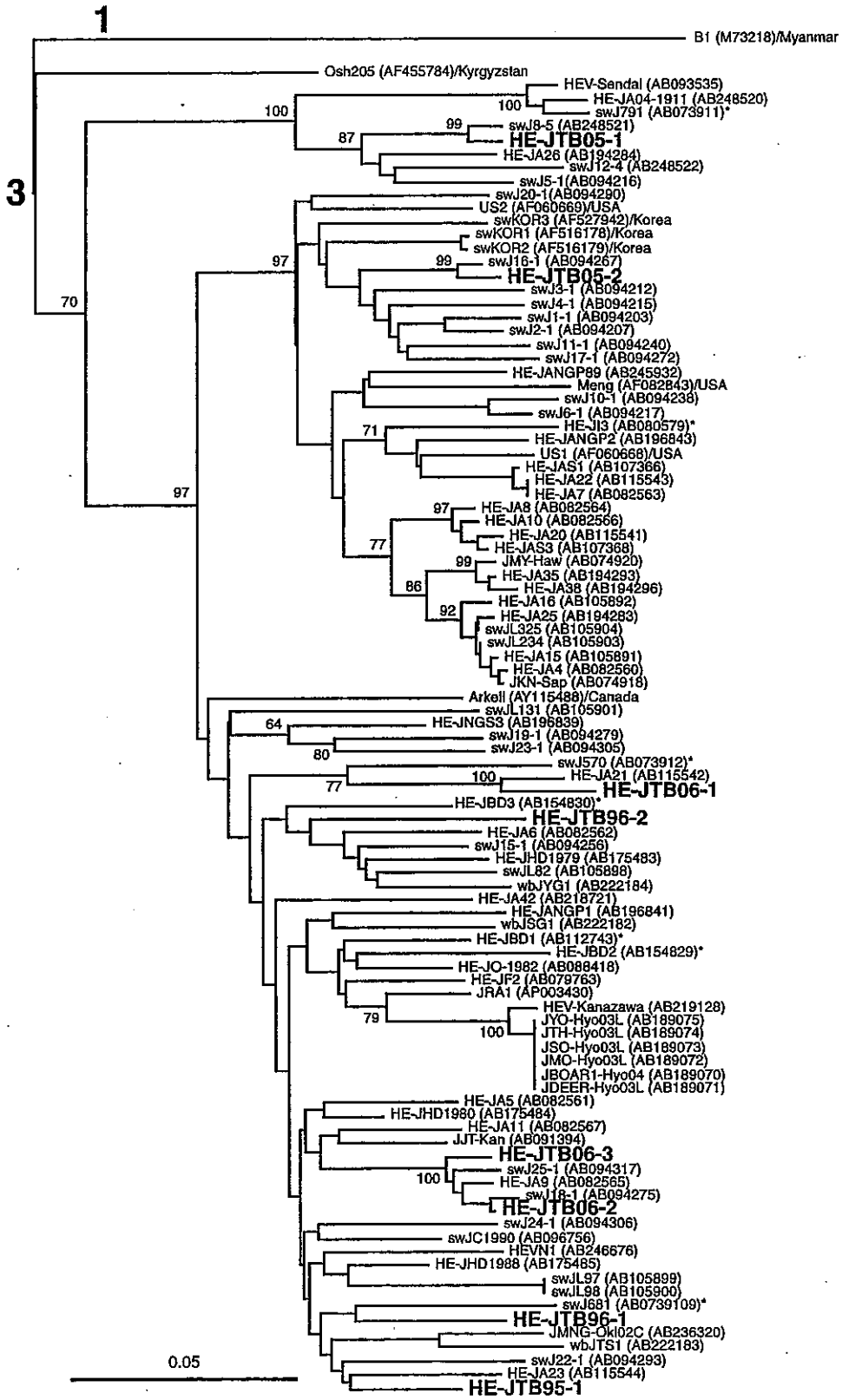
1
3
B1 (M73218)/Myanmar
Osh205 (AF455784)/Kyrgyzstan
HEV-Sendai (AB093535)
HE-JA04-1911 (AB248520)
swJ791 (AB073911)*
swJ8-5 (AB248521)
HE-JTB05-1
HE-JA26 (AB194284)
swJ12-4 (AB248522)
swJ5-1(AB094216)
swJ20-1(AB094290)
US2 (AF060669)/USA
swKOR3 (AF527942)/Korea
swKOR1 (AF516178)/Korea
swKOR2 (AF516179)/Korea
swJ16-1 (AB094267)
HE-JTB05-2
swJ3-1 (AB094212)
swJ4-1 (AB094215)
swJ1-1 (AB094203)
swJ2-1 (AB094207)
swJ11-1 (AB094240)
swJ17-1 (AB094272)
HE-JANGP89 (AB245932)
Meng (AF082843)/USA
swJ10-1 (AB094238)
swJ6-1 (AB094217)
HE-JI3 (AB080579)*
HE-JANGP2 (AB196843)
US1 (AF060668)/USA
HE-JAS1 (AB107366)
HE-JA22 (AB115543)
HE-JA7 (AB082563)
HE-JA8 (AB082564)
HE-JA10 (AB082566)
HE-JA20 (AB115541)
HE-JAS3 (AB107368)
JMY-Haw (AB074920)
HE-JA35 (AB194293)
HE-JA38 (AB194296)
HE-JA16 (AB105892)
HE-JA25 (AB194283)
swJL325 (AB105904)
swJL234 (AB105903)
HE-JA15 (AB105891)
HE-JA4 (AB082560)
JKN-Sap (AB074918)
Arkell (AY115488)/Canada
swJL131 (AB105901)
HE-JNGS3 (AB196839)
swJ19-1 (AB094279)
swJ23-1 (AB094305)
swJ570 (AB073912)*
HE-JA21 (AB115542)
HE-JTB06-1
HE-JBD3 (AB154830)*
HE-JTB96-2
HE-JA6 (AB082562)
swJ15-1 (AB094256)
HE-JHD1979 (AB175483)
swJL82 (AB105898)
wbJYG1 (AB222184)
HE-JA42 (AB218721)
HE-JANGP1 (AB196841)
wbJSG1 (AB222182)
HE-JBD1 (AB112743)*
HE-JBD2 (AB154829)*
HE-JO-1982 (AB088418)
HE-JF2 (AB079763)
JRA1 (AP003430)
HEV-Kanazawa (AB219128)
JYO-Hyo03L (AB189075)
JTH-Hyo03L (AB189074)
JSO-Hyo03L (AB189073)
JMO-Hyo03L (AB189072)
JBOAR1-Hyo04 (AB189070)
JDEER-Hyo03L (AB189071)
HE-JA5 (AB082561)
HE-JHD1980 (AB175484)
HE-JA11 (AB082567)
JJT-Kan (AB091394)
HE-JTB06-3
swJ25-1 (AB094317)
HE-JA9 (AB082565)
swJ18-1 (AB094275)
HE-JTB06-2
swJ24-1 (AB094306)
swJC1990 (AB096756)
HEVN1 (AB246676)
HE-JHD1988 (AB175485)
swJL97 (AB105899)
swJL98 (AB105900)
swJ681 (AB0739109)*
HE-JTB96-1
JMNG-Okl02C (AB236320)
wbJTS1 (AB222183)
swJ22-1 (AB094293)
HE-JA23 (AB115544)
HE-JTB95-1
0.05

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2 region (412 nt) of 65 HEV isolates, using a genotype 1 HEV (M73218) as an outgroup. In addition to the HE-JTB94-1, HE-JTB98-1, and HE-JTB98-2 isolates found in the present study, which are indicated in bold type, 61 reported HEV isolates of genotype 4, whose common 412-nt sequence is known, are included for comparison. The reported isolates are indicated with the accession no. followed by the name of the country where it was isolated (non-Japanese origin only). An asterisk denotes human HEV strains that were isolated in the same prefecture as those obtained in the present study. Bootstrap values are indicated for the major nodes as a percentage obtained from 1000 resamplings of the data

mans, swine and wild boars, supporting the indigenous nature of these 8 blood donor isolates (Fig. 1). On the other hand, the remaining three HEV isolates obtained in the present study (HE-JTB94-1, HE-JTB98-1 and HE-JTB98-2) were close to the prototype genotype 4 isolate (T1) with nu-

cleotide identities of 84.7–87.6%, and were only 79.9–81.8, 77.7–78.4, and 79.9–81.3% similar to the B1 isolate of genotype 1, MEX-14 isolate of genotype 2, and JRA1 isolate of genotype 3, respectively, suggesting that the HE-JTB94-1, HE-JTB98-1 and HE-JTB98-2 isolates belong to genotype 4. The phylogenetic tree constructed based on the common 412-nt ORF2 sequence confirmed that the 3 HEV isolates obtained in the present study segregated to genotype 4 (Fig. 2). Of note, the HE-JTB94-1 and HE-JTB98-1 isolates segregated into two distinct clusters consisting of Japanese HEV strains, each with a bootstrap value of 100%, but HE-JTB98-2 segregated into a cluster comprising the Chinese HEV strains that had been recovered from a Chinese patient with autochthonous hepatitis E (accession no. EF077630) and Japanese patients with hepatitis E who had traveled to China (AB197673–AB197674), suggesting that the HE-JTB98-2 isolate may be of China origin.

Discussion

This study examined the prevalence of ongoing subclinical HEV infection among 4019 apparently healthy blood donors with an elevated ALT level of ≥61 IU/l who donated blood during the last 16 years at a Japanese Red Cross Blood Center located in the northern part of mainland Honshu of Japan. As for the geographical distribution of hepatitis E in Japan, it was reported that there was wide variation, with a higher prevalence in the northern part of Japan (Hokkaido Island and the northern part of mainland Honshu) [1, 30], suggesting that the results obtained in the present study cannot simply be generalized for the whole country. However, our study corroborated the previous study by Tanaka et al. [51], who reported that, based on the age-specific distribution of anti-HEV IgG in 1974, 1984, and 1994, exposure to HEV remained constant between 1974 and 1994 in Metropolitan Tokyo, Japan. The prevalence of clinical HEV infection among patients with acute hepatitis remained unchanged during the period from 1989 to 2005 in a city hospital in Aichi Prefecture, which is located in the central part of Honshu Island of Japan [29]. Therefore, our present study may represent the recent trends of HEV infection, at least in the northern and central parts of mainland Honshu of Japan, where hepatitis E is low-endemic [1].

The presence of anti-HEV IgG most likely reflects past subclinical HEV infection. The present study revealed that the prevalence of anti-HEV IgG among blood donors with ALT of ≥61 IU/l between 2004 and 2006 was similar to that in 1998 (5.3 vs. 5.2%), and that the prevalence of anti-HEV IgG among blood donors with ALT of ≥201 IU/l was comparable among the three year groups of 1991–1995, 1996–1999 and 2004–2006 (5.8, 4.3, and 6.6%, respectively) as well as that of 2002–2003 (4.1% or 23/560) [7]. Longitudinal seroepidemiological studies on transiently infected individuals suggested that anti-HEV IgG persisted much longer than expected, i.e., for more than 20 years [27, 28]. Even a low titer of anti-HEV IgG may reflect past subclinical HEV infection and has been detected in a significant proportion of healthy individuals not only in Japan but also in the United States and European countries [8, 16, 33, 36, 40, 51]. Therefore, in studies in which anti-HEV IgG is assayed at a single time point in each individual, it may be hard to specify when individuals with anti-HEV IgG contracted HEV infection and how prevalent de novo subclinical HEV infection was during a particular period.

In the present study, the genomic RNA of HEV was detected in a total of 11 donors with an ALT level of 63–2598 IU/l among the 4019 donors tested. When stratified by the year group of blood collection, the prevalence of HEV viremia among blood donors with ALT of ≥61 IU/l between 2004 and 2006 was comparable to that in 1998 (0.2 vs. 0.3%), and the prevalence of HEV viremia among blood donors with ALT of ≥201 IU/l was not statistically different among the three year groups of 1991–1995, 1996–1999, and 2004–2006 (1.3, 3.4, and 3.3%, respectively), or from that of 2002–2003 (4.4% or 1/23) [7], suggesting that de novo subclinical HEV infection occurred at an almost constant rate during the last 16 years in Tochigi Prefecture, Japan. In industrialized countries including Japan, maintenance of good hygiene of the water supply and sewage systems made the likelihood of waterborne infection of hepatitis A virus (HAV) extremely low [13]. However, our observations are consistent with the notion that transmission of HEV would not

be prevented by only improvement of sanitary conditions, despite the lower infectivity and transmissibility of HEV than HAV [36].

Domestically acquired hepatitis E has been reported in industrialized countries including the United States and European countries since 1997 [2, 5, 10, 15, 21, 34, 35, 37, 55]. However, only a limited number of sporadic cases of acute hepatitis E have been reported in the United States and European countries, and the changing profiles of clinical and subclinical HEV infection have not been studied in these countries. In Japan, clinical hepatitis E is rare compared with clinical hepatitis A but is occurring more frequently than previously thought [30, 33], where the first case of autochthonous hepatitis E was reported in 2001 [43], and presumably indigenous HEV strains have been recovered from individuals who contracted HEV infection in the 1970s [27, 28]. It remains unknown, however, why the prevalence of domestic HEV infection has remained stable during the last few decades in Japan. It has recently been suggested that zoonotic foodborne transmission of HEV from domestic pigs and wild boars to humans plays an important role in the occurrence of cryptic hepatitis E in Japan, where Japanese people have distinctive habits of eating raw fish (sushi or sashimi) and, less frequently, uncooked or undercooked meat (including the liver and colon/intestine of animals) [17, 23, 31, 41, 57]. Of note, we found a high prevalence of swine anti-HEV antibodies and a high HEV viremia rate among Japanese pigs [46, 48]. The majority of patients with sporadic acute hepatitis E in Hokkaido had a history of consuming grilled or undercooked pig liver and/or intestine approximately 2–8 weeks prior to the onset of hepatitis E [31]. Pig liver specimens from 7 (1.9%) of 363 packages sold in local grocery stores in Hokkaido had detectable HEV RNA [57]. These results strongly suggest that consumption of undercooked pig liver/intestine is a potential risk factor for HEV infection. Transfusion-associated hepatitis E has also been reported in Japan [22, 27]. Recently, of interest, it was reported that HEV RNA was detected in bivalves called Yamato-Shijimi (*Corbicula japonica*) obtained from Japanese rivers, indicating that HEV contaminates river water in Japan [18]. However, the mode of HEV transmission in the 11 viremic donors in the present study was unclear. Further studies are needed to elucidate the mode(s) of clinical and subclinical HEV infection in the general population of Japan including Tochigi Prefecture.

As the 11 viremic donors identified in the present study had an elevated ALT level, the blood from the 11 donors was not used for transfusion, suggesting that ALT testing helps prevent transfusion-transmitted HEV infection. As one of the 11 infected donors had only a slightly elevated ALT level of 63 IU/l, it seems likely that even donors with a normal ALT level ($\leq$60 IU/l) may have detectable HEV RNA. The prevalence of HEV RNA decreased with the ALT level and was significantly less frequent among the 3000 donors with ALT of 61–100 IU/l than among the 93 donors with ALT of $\geq$201 IU/l (0.067 vs. 4.3%, $P<0.0001$) in 1998 and 2004–2006. Although the number of donors tested was limited, it is reasonable to speculate that the prevalence of ongoing HEV infection among donors with a normal ALT level may be less than 0.067% in Tochigi Prefecture. The proportion of such donors may be very small or negligible, particularly in the southern part of Japan, where only 1.7% (9/527) of blood donors with ALT of $\geq$61 IU/l had anti-HEV IgG [7].

Reflecting the polyphyletic nature of human and animal HEV isolates of Japanese origin [30, 33, 44], the HEV isolates recovered from the 11 viremic donors in the present study differed by 1.7–22.8% from each other and segregated into genotype 3 or 4. Ten human HEV strains of genotype 3 (HE-JI3 [AB080579], HE-JBD1 [AB112743], HE-JBD2 [AB154829], HE-JBD3 [AB154830], and 6 unpublished isolates) have been isolated in the same prefecture as that of the 11 viremic donors and shared identities ranging from 80.6 to 99.8% with the 8 genotype 3 HEV isolates obtained in the present study. As for human HEV strains of genotype 4, two strains (HE-JI4 [AB080575] and HE-JK4 [AB099347]) isolated in the same prefecture shared 87.9–99.3% identities with the 3 genotype 4 HEV isolates obtained in the present study. These results further support the marked heterogeneity of the HEV genome and its wide distribution in Japan, even within a certain prefecture in this country.