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# Epidemiological and clinical study of sporadic acute hepatitis E caused by indigenous strains of hepatitis E virus in Japan compared with acute hepatitis A

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***Background.*** We compared acute hepatitis E (AH-E) and acute hepatitis A (AH-A) to investigate the epidemiology, clinical features, and prognosis of AH-E caused by an indigenous hepatitis E virus (HEV) in Japan. ***Methods.*** We enrolled 58 patients diagnosed with AH-A or AH-E (32 men and 26 women; age, 20–72 years) from December 1997 to October 2002. Phylogenetic analysis of the partial 412-nucleotide sequence of open reading frame (ORF) 2 was performed in patients with AH-E. ***Results.*** Regarding the geographic distribution of the HEV genotype, genotype III was principally distributed in Honshu Island, and genotype IV in Hokkaido Island ($P = 0.0034$). The phylogenetic analysis of the ORF2 region revealed that there were significant geographic differences in the distribution of the HEV strains in Japan, with some strains being widespread and some, localized. In comparison with AH-A patients, those with AH-E were older (56.1 ± 10.6 vs 45.9 ± 10.8 years; $P = 0.0017$). The proportion of males among patients with AH-E was significantly higher ($P = 0.0001$). Pyrexia was often observed in AH-A, and malaise in AH-E. Laboratory data indicate that AH-E induces a weak immunological reaction, whereas jaundice appears earlier in AH-E than in AH-A. One patient with AH-E died of acute hepatic failure, but none of those with AH-A died during the study period. ***Conclusions.*** Our results suggest that there are geographical differences between HEV strains in Japan, and that AH-E is more common in males and older patients than AH-A. Laboratory data indicate a weak immunological reaction and early appearance of jaundice in AH-E.

**Key words:** acute hepatitis E, epidemiology, hepatitis A, phylogenetic analysis

## Introduction

The hepatitis E virus (HEV) is a small, non-enveloped, icosahedral, positive-sense, single-strand RNA virus. HEV is responsible for the majority of cases of what was previously called enterically transmitted non-A, non-B hepatitis. Hepatitis E is endemic in many subtropical and tropical areas. In these areas, hepatitis E occurs both epidemically and sporadically. Hepatitis E is a self-limiting disease of varying severity, presenting as acute, icteric hepatitis, with clinical and morphological findings similar to those of hepatitis A. Recent studies have found immunoglobulin G (IgG) to HEV (anti-HEV IgG) in several wild and domestic animal species native to developing and industrialized countries.[1] Molecular evidence for natural HEV infection in swine has been reported for HEV-endemic and -nonendemic countries worldwide.[2–8] Novel HEV strains from nonendemic areas were detected in patients in the United States, Taiwan, Greece, Italy, Spain, Austria, and Argentina.[4,5,9–11] Now, it is thought that HEV may be more widespread than previously thought.

In Japan, HEV infection rarely occurs, and most, if any, cases of hepatitis E observed thus far have been regarded as imported cases of hepatitis.[12,13] However, the seroprevalence of anti-HEV IgG in healthy individuals in Japan was reported to range from 1.9% to 14.1%, depending on the geographic area.[14] In addition,

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an indigenous HEV strain of genotype III (strain JRA1) has been isolated from a Japanese patient with acute hepatitis who had never been abroad,[15] and a swine HEV strain (strain swJ570) with the highest degree of similarity to the JRA1 isolate among the known HEV isolates has been isolated from domesticated pigs in Japan, although their entire genomes shared only 89% identity.[16] A recent study revealed that polyphyletic HEV strains of genotypes III and IV cocirculate in Japan and contribute to the development of sporadic acute hepatitis of non-ABC etiology, with higher prevalences in males, in those over 40 years of age, and in patients living in the northern part of Japan.[17] However, there are few cases of sporadic hepatitis E in nonendemic countries, including Japan, compared with endemic countries, where outbreaks of hepatitis E may often occur. Therefore, the clinical features of hepatitis E caused by indigenous HEV strains in Japan have not been sufficiently studied as compared with those in endemic countries. In the present study, we investigated the epidemiology, clinical features, and prognosis of acute hepatitis E (AH-E) in Japan compared with hepatitis A (AH-A), which shares similar transmission routes and clinical manifestations with AH-E.

## Patients and methods

### *Patients*

A total of 58 patients (32 men and 26 women; age, 20–72 years) diagnosed with AH-A or AH-E from December 1997 to October 2002 were enrolled in the present study. We retrospectively investigated patients with hepatitis E that occurred in Japan during this 5-year period, and we asked each hospital that had the patients to join the co-operative study. The patients with AH-E were treated at six city or university hospitals in Sapporo (Hokkaido Island), and in Iwate, Miyagi, Fukushima, Yamanashi, and Nagano in mainland Honshu, Japan. In all these patients the hepatitis E was non-imported. Patients with AH-A were all admitted to the university hospitals of Iwate Medical University. These patients were from the same general geographic region as the hospital at which they were treated. They were all negative for hepatitis B surface antigen (HBsAg), anti-hepatitis B virus (HBV) core immunoglobulin M (IgM), and anti-hepatitis C virus (anti-HCV). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Ethics Committee of each institution, and informed consent was obtained from each patient. Serum samples were obtained and stored at −20°C or below until assay.

Forty-one patients (16 men, 25 women; age, 20–26 years) with serum anti-hepatitis A virus (HAV) IgM (HAVAB-M; Abbott Laboratories, North Chicago, IL, USA) were diagnosed with AH-A. Seventeen patients (16 men, 1 woman; age, 42–72 years) were diagnosed with AH-E, based on positivity for IgM class antibodies to HEV (anti-HEV IgM), determined by serological study, as well as showing HEV RNA detected by reverse transcription-polymerase chain reaction (RT-PCR).

### *Epidemiological study and laboratory examinations*

All patients enrolled in the study had their detailed history taken, including general data such as age, sex, time of onset of illness, travel history before onset of illness, history of blood transfusion, alcohol intake, medicine use, and disease complication. As the incubation periods of AH-A and AH-E are approximately 2 to 8 weeks and 2 to 10 weeks, respectively, travel abroad within 3 months before the onset of illness was regarded as having a travel history. In laboratory examinations, white blood cells, atypical lymphocytes (%), total bilirubin (T. Bil), thymol turbidity test (TTT) value, zinc sulfate turbidity test (ZTT) value, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl-transpeptidase (γ-GTP), alkaline phosphatase (ALP), and IgM were measured, using a sequential multiautoanalyzer at each hospital.

### *Detection of anti-HEV IgM*

Anti-HEV IgM was measured using purified recombinant HEV open reading frame (ORF) 2 protein, according to a procedure described by Mizuo et al.[17] Briefly, wells of microplates were coated with purified virus-like particles of HEV expressed by a recombinant baculovirus. Fifty microliters of each sample was added to each well at a dilution of 1:100 in saline containing 40% calf serum. The microplates were incubated at room temperature for 1 h with gentle agitation and were then washed five times with washing buffer. Fifty microliters of phosphate-buffered saline containing 25% fetal bovine serum and peroxidase-conjugated mouse monoclonal anti-human IgM was added to each well. The microplates were incubated at room temperature for 1 h with gentle agitation and then washed five times with washing buffer. Fifty microliters of tetramethylbenzidine (TMB) soluble reagent as substrate was added to each well. The plate was incubated at room temperature for 10 min in the dark, and then 50 μl of TMB stop buffer was added to each well. The optical density (OD) of each sample was read at 450 nm. Test samples with ODs equal to or greater than the cutoff value were considered positive for anti-HEV IgM.

*Detection of HEV RNA and sequence analysis of PCR products*

Serum HEV RNA was detected by nested RT-PCR analysis as previously reported.[17] Briefly, total RNA was extracted from the serum sample with guanididium thiocyanate and phenol-chloroform, using TRIZOL LS reagent (Invitrogen, Groningen, The Netherlands). The RNA preparation was reverse-transcribed with SuperScript II RNase $H^-$ reverse transcriptase (Invitrogen) and was then subjected to nested PCR with ORF2-specific primers. The size of the amplification product of the first-round PCR was 506 base pairs (bp), and that of the second-round PCR was 458 bp.

The amplification products were electrophoresed on a 1.5% (wt/vol) NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME, USA) stained with ethidium bromide, and photographed under UV light. The RT-PCR was performed in duplicate, and reproducibility was confirmed. The amplification products were directly sequenced on both strands. Sequence analysis was performed as previously reported.[17] A phylogenetic tree was constructed by the neighbor-joining method, based on the partial nucleotide sequence of the open reading frame (ORF) 2 region (412 nucleotides [nt]).[18] Bootstrap values were determined on 1000 resamplings of data sets.[19] The geographic origins and the GenBank accession numbers of the nucleotide sequences of the HEV strains used in the phylogenetic analysis were as follows: JRA1 (Japan, AP003430), HE-JF2 (Japan, AB079763), HE-JO-1982 (Japan, AB088418), swJ681 (Japan, AB073912), swJ570 (Japan, AB073912), US1 (United States, AF060668), HE-JI3 (Japan, AB080579), JKN-Sap (Japan, AB074918), JMY-Haw (Japan, AB074920), HE-JA10 (Japan, AB089824), US2 (United States, AF060669), swJ791 (Japan, AB073911), HE-JF1 (Japan, AB079762), JAK-Sai (Japan, AB074915), JKK-Sap (Japan, AB074917), HE-JI4 (Japan, AB080575), HE-JF3 (Japan, AB079764), T1 (China, AJ272108), C1 (China, D11092), C2 (China, L25547), C3 (China, M94177), C4 (China, D11093), C5 (China, L08816), P1 (Pakistan, M80581), P2 (Pakistan, AF185822), I1 (India, X98292), I2 (India, X99441), I3 (India, AF076239), I4 (India, AF459438), B1 (Myanmar, M73218), B2 (Myanmar, D10330), Ne1 (Nepal, AF051830), and MEX-14 (Mexico, M74506).

*Statistical analysis*

We used $\chi^2$ analysis, Fisher's exact test, Student's *t*-test, and Mann-Whitney's *U*-test where appropriate in this study. All significant data were two-tailed, and a *P* value of less than 0.05 was considered significant.

## Results

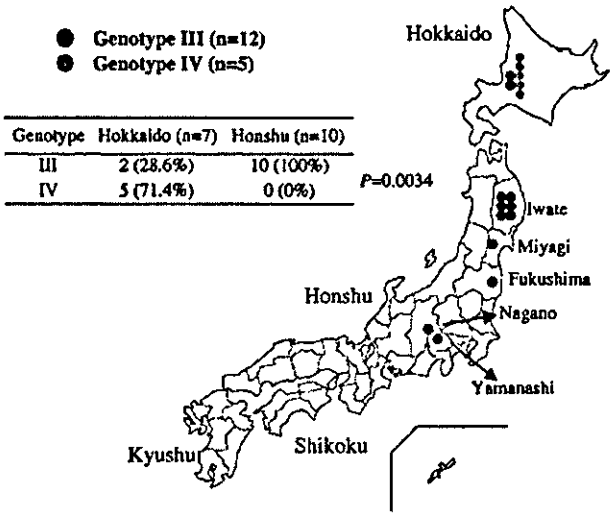
*Geographic distribution of HEV according to HEV genotype in patients with AH-E, and phylogenetic analysis of the partial 412-nt sequence of the ORF2 region*

Ten HEV isolates, from patients 1, 2, 3, 4, 8, 9, 10, 11, 15, and 16, have already been reported by Mizuo et al.,[17]

**Table 1.** Profiles of 17 patients with AH-E

| Patient no. | Age (years) | Sex | Onset | Location | HEV genotype | Name of HEV isolate |
|---|---|---|---|---|---|---|
| 1 | 55 | M | Dec., 1997 | Hokkaido | IV | HE-JA1[a] |
| 2 | 71 | M | Aug., 1998 | Hokkaido | IV | HE-JA2[a] |
| 3 | 42 | M | Oct., 1998 | Hokkaido | IV | HE-JA3[a] |
| 4 | 44 | M | Jan., 2000 | Hokkaido | III | HE-JA4[a] |
| 5 | 46 | M | May, 2001 | Hokkaido | IV | HE-JA13 |
| 6 | 72 | M | Aug., 2002 | Hokkaido | III | HE-JA16 |
| 7 | 64 | M | Sep., 2002 | Hokkaido | IV | HE-JF4 |
| 8 | 48 | M | Aug., 1998 | Iwate | III | HE-JA5[a] |
| 9 | 47 | F | May, 1999 | Iwate | III | HE-JA6[a] |
| 10 | 72 | M | Mar., 2001 | Iwate | III | HE-JA7[a] |
| 11 | 56 | M | Jul., 2001 | Iwate | III | HE-JA8[a] |
| 12 | 62 | M | Sep., 2002 | Iwate | III | HE-JA21 |
| 13 | 71 | M | Oct., 2002 | Iwate | III | HE-JA22 |
| 14 | 54 | M | Jun., 2002 | Miyagi | III | HEV-Sendai[b] |
| 15 | 45 | M | Jan., 2001 | Fukushima | III | HE-JA9[a] |
| 16 | 50 | M | Nov., 2001 | Yamanashi | III | HE-JA11[a] |
| 17 | 55 | M | Jul., 2002 | Nagano | III | HE-JA23 |

AH-E, acute hepatitis E; HEV, hepatitis E virus
[a] These isolates have been reported previously by Mizuo et al.[17]
[b] This isolate has been reported by Yajima et al.[20]




| Genotype | Hokkaido (n=7) | Honshu (n=10) |
|---|---|---|
| III | 2 (28.6%) | 10 (100%) |
| IV | 5 (71.4%) | 0 (0%) |

**Fig. 1.** Geographic distribution of patients with acute hepatitis E (AH-E) in Japan according to hepatitis E virus (HEV) genotype

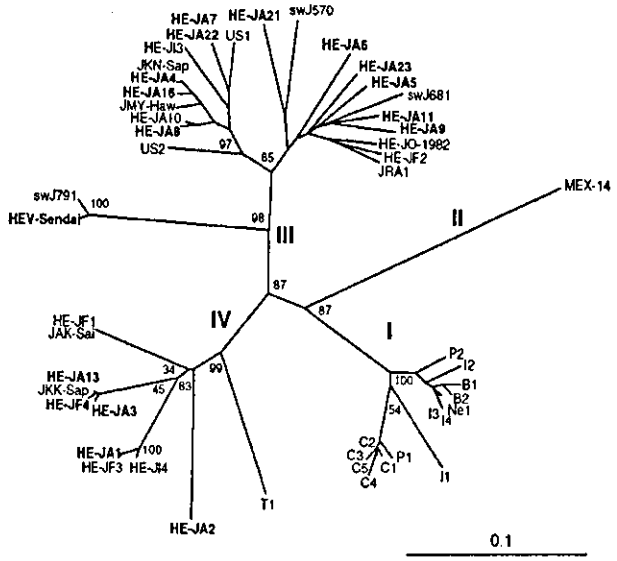



**Fig. 2.** Phylogenetic tree constructed by the neighbor-joining method, based on the partial nucleotide (nt) sequence of the open reading frame (ORF) 2 region (412 nt) of 50 human and swine isolates. In addition to 26 reported human and swine HEV isolates of genotypes I to IV whose entire or nearly entire sequences are known, seven reported isolates of genotype III or IV whose partial sequences of 412, 421, or 436 nt have been determined were included for comparison. They were deposited under accession nos. AB073910–AB073912, AB074915, AB074917, AB074918, AB074920, AB079762–AB079764, AB080575, AB080759, AB088418, AB089824, AF051830, AF060668, AF060669, AF076239, AF185822, AF459438, AJ272108, AP003430, D10330, D11092, D11093, L08816, L25547, M73218, M74506, M80581, M94177, X98292, and X99441. The 17 HEV isolates from this study are indicated in *boldface*. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings

and an HEV isolate from patient 14 has been reported by Yajima et al.[20] HEV isolates from 5 patients were classified as genotype IV and those from 12 patients were classified as genotype III (Table 1).

According to the geographic distribution of HEV genotype (Fig. 1), the HEV isolates from ten patients in mainland Honshu (six in Iwate and one each in Miyagi, Fukushima, Nagano, and Yamanashi) were all classified as genotype III. However, of seven HEV isolates in Hokkaido, only two (28.6%) were classified as genotype III, and the others were classified as genotype IV. There was a significant geographic difference in the HEV genotype between Honshu Island and Hokkaido Island ($P = 0.0034$).

Phylogenetic analysis of the partial 412-nt sequence of the ORF2 region revealed that polyphyletic HEV strains of genotypes III and IV exist in Japan (Fig. 2). However, regardless of a different year of onset, nucleotide identity of the 412-nt sequence of the ORF2 region between HE-JA7 and HE-JA22 was 100%, and these two HEV strains were isolated from patients living in the same location in Iwate. Furthermore, these two isolates were found to be the most homologous to the human strain in the United States (US1).[8] For genotype IV, HE-JA3 from patient 3, HE-JF4 from patient 7, and HE-JA13 from patient 5, all of whom lived in Hokkaido, closely resembled JKK-Sap—isolated from a patient who also lived in Hokkaido—that has been reported previously.[21] On the other hand, HE-JA1, isolated from patient 1, who lived in Hokkaido, shared 100% nucleotide identity with the HE-JF3 isolate, which was isolated in 2002 from a patient who lived in Iwate, on Honshu Island.[22]

*Comparison of clinical features between AH-A and AH-E*

The month of onset of illness was investigated in patients with AH-A and those with AH-E (Fig. 3). AH-A onset in Japan showed a normal distribution from January to December, and AH-A occurred generally in winter and spring, particularly in March. However, the onset of disease was distributed almost equally over the year in patients with AH-E, and there was no particular season when AH-E occurred predominantly.

A comparison of clinical manifestations between AH-A and AH-E is shown in Table 2. As for the profile of patients, the mean age of AH-A patients was 45.9 years, whereas that of AH-E patients was 56.1 years. Patients with AH-E were significantly older at time of onset than those with AH-A ($P = 0.0017$). Moreover, the proportion of males among patients with AH-E was significantly higher than that among AH-A patients ($P$

= 0.0001). However, there were no significant differences between the groups in history of blood transfusion, medication, and disease complication.

According to clinical symptoms, pyrexia (>38°C) was present in 73.2% of patients with AH-A, but in only 41.2% of patients with AH-E, and the difference was significant ($P$ = 0.0210). Malaise was noted in 65.9% of patients with AH-A, but in 100% of patients with AH-E, and the difference was significant ($P$ = 0.0055). However, there were no significant differences between the groups in other symptoms, such as flu-like prodromes (including myalgia, arthalgia, or headache), nausea or vomiting, abdominal pain, pruritus, and diarrhea.

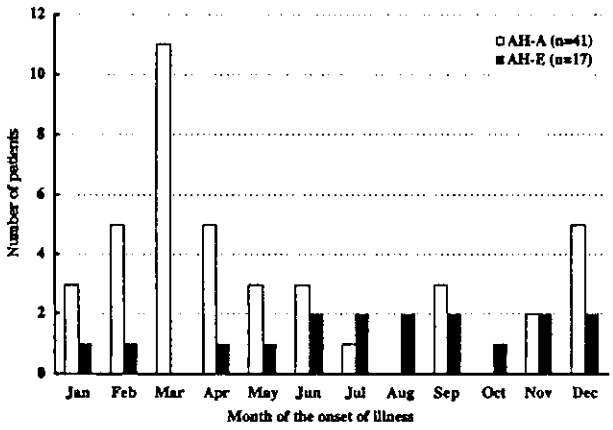



**Fig. 3.** Distribution of the onset of illness in patients with AH-A or AH-E in a year

As for physical findings, lymphadenopathy was observed in only 19.5% of patients with AH-A, whereas it was not observed in any patients with AH-E. However, there was no significant difference between the groups in lymphadenopathy, and there were no differences between the groups in other physical findings, such as jaundice, hepatomegaly, splenomegaly, exanthema, and edema.

*Comparison of laboratory data between patients with AH-A and those with AH-E*

We investigated laboratory data on admission, as well as investigating peak values, and we compared the mean values for the data between the study groups (Table 3). The percentage of atypical lymphocytes was higher in AH-A patients on admission (7.50% vs 2.07%; $P$ = 0.0288). The T. Bil level was lower in AH-A patients on admission (4.27 vs 7.33 mg/dl; $P$ = 0.0032), whereas the peak values were the same in the two groups. ZTT values were markedly higher in AH-A

**Table 2.** Patient demographics and clinical characteristics of AH-A and AH-E

| Characteristics | AH-A ($n$ = 41) | AH-E ($n$ = 17) | $P$ |
|---|---|---|---|
| Profile | | | |
| Age (years) | | | |
| Mean ± SD | 45.9 ± 10.8 | 56.1 ± 10.6 | 0.0017 |
| Sex | | | |
| Male/Female | 16/25 | 16/1 | 0.0001 |
| History of traveling abroad within 3 months before the onset of illness; no. (%) | 0 (0) | 0 (0) | 0.9999< |
| History of blood transfusion within 3 months before the onset of illness; no. (%) | 4 (9.8) | 0 (0) | 0.3100 |
| History of alcohol intake (over 60 g ethanol daily); no. (%) | 12 (29.3) | 5 (29.4) | 0.8562 |
| Medication history; no. (%) | 15 (36.6) | 4 (23.5) | 0.3349 |
| Disease complication; no. (%) | 17 (41.5) | 7 (41.2) | 0.9839 |
| Symptoms; no. (%) | | | |
| Pyrexia (>38°C) | 30 (73.2) | 7 (41.2) | 0.0210 |
| Malaise | 27 (65.9) | 17 (100) | 0.0055 |
| Flu-like prodrome | 17 (41.5) | 10 (58.8) | 0.2276 |
| Nausea or vomiting | 17 (41.5) | 7 (41.2) | 0.9839 |
| Abdominal pain | 9 (22.0) | 2 (11.8) | 0.4796 |
| Pruritus | 6 (14.6) | 2 (11.8) | 0.9999< |
| Diarrhea | 1 (2.4) | 0 (0) | 0.9999< |
| Physical findings; no. (%) | | | |
| Jaundice | 34 (82.9) | 15 (88.2) | 0.9999< |
| Hepatomegaly | 10 (24.4) | 4 (23.5) | 0.9999< |
| Splenomegaly | 8 (19.5) | 3 (17.6) | 0.9999< |
| Lymphadenopathy | 8 (19.5) | 0 (0) | 0.0900 |
| Exanthema | 1 (2.4) | 2 (11.8) | 0.9999< |
| Edema | 0 (0) | 0 (0) | 0.9999< |

AH-A, acute hepatitis A; AH-E, acute hepatitis E

**Table 3.** Comparison of laboratory data on admission, and peak values, between AH-A and AH-E

| Variable | Values on admission | | | Values at peak | | |
|---|---|---|---|---|---|---|
| | AH-A (*n*) | AH-E (*n*) | *P* | AH-A (*n*) | AH-E (*n*) | *P* |
| White blood cells (per mm³) | 5550 ± 2124 (41) | 5402 ± 1615 (17) | 0.7981 | 8620 ± 4088 (41) | 9013 ± 8368 (17) | 0.8104 |
| Atypical lymphocytes (%) | 7.50 ± 9.81 (41) | 2.07 ± 2.40 (17) | 0.0288 | 7.81 ± 9.56 (41) | 3.28 ± 3.60 (17) | 0.0643 |
| Total bilirubin (mg/dl) | 4.27 ± 1.71 (41) | 7.33 ± 5.84 (17) | 0.0032 | 8.88 ± 4.50 (41) | 10.89 ± 8.31 (17) | 0.2377 |
| TTT (KU) | 9.54 ± 4.19 (40) | 11.58 ± 11.40 (15) | 0.3319 | 15.79 ± 3.93 (40) | 17.54 ± 14.83 (15) | 0.4917 |
| ZTT (KU) | 15.86 ± 6.08 (41) | 11.98 ± 6.94 (16) | 0.0420 | 26.17 ± 8.50 (41) | 14.24 ± 7.26 (16) | <0.0001 |
| AST (IU/l) | 3278 ± 4126 (41) | 1689 ± 1667 (17) | 0.1315 | 3384 ± 4117 (41) | 1842 ± 1575 (17) | 0.1410 |
| ALT (IU/l) | 3486 ± 2673 (41) | 2204 ± 1417 (17) | 0.0674 | 3775 ± 2682 (41) | 2407 ± 1247 (17) | 0.0496 |
| γ-GTP (IU/l) | 340 ± 210 (41) | 363 ± 290 (17) | 0.7350 | 400 ± 236 (41) | 380 ± 294 (17) | 0.7819 |
| ALP (IU/l) | 610 ± 208 (41) | 659 ± 211 (17) | 0.4117 | 730 ± 223 (41) | 692 ± 194 (17) | 0.5415 |
| IgM (mg/dl) | 422.1 ± 209.4 (39) | 237.4 ± 129.4 (13) | 0.0044 | 509.7 ± 204.1 (41) | 237.6 ± 129.3 (13) | <0.0001 |

Plus-minus values are means ± SDs
AH-A, acute hepatitis A; AH-E, acute hepatitis E; TTT, thymol turbidity test; ZTT, zinc sulfate turbidity test; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl-transpeptidase; ALP, alkaline phosphatase; IgM, immunoglobulin M

patients, both on admission (15.86 vs 11.98 KU; $P = 0.042$) and at peak (26.17 vs 14.24 KU; $P > 0.0001$). The peak ALT level was higher in AH-A patients (3775 vs 2407 IU/l; $P = 0.0496$). The IgM titers were markedly higher in AH-A patients, both on admission (422.1 vs 237.4 mg/dl; $P = 0.0044$) and at peak (509.7 vs 237.6 mg/dl; $P = 0.0001$).

However, no significant differences between the groups were found in other laboratory variables; namely, the white cell count, and γ-GTP and ALP levels.

### *Prognosis of AH-A and AH-E*

One patient with AH-E (patient 7), who lived in Hokkaido, died of acute hepatic failure during the study period, but none of the AH-A patients died during the study period (Table 4). In the 49 patients (34 with AH-A, 15 with AH-E) exhibiting jaundice, the period between the onset of illness and the decrease in the T. Bil level to less than 2.0 mg/dl was the same in both groups. In addition, there was no significant difference between the two groups in the period from the onset of illness to the decrease in the ALT level to less than 40 IU/l. In the patients with jaundice, the number of days from admission to the peak T. Bil level in the AH-E group was distributed as follows: 8 patients (53.3%) within 7 days; 5 (33.3%) from 8 to 14 days, and 2 (13.3%) at more than 15 days. In the AH-A group, the number of days from admission to the peak T. Bil, level was distributed as follows: 4 patients (11.8%) within 7 days, 20 (58.8%) from 8 to 14 days, and 10 (29.4%) at more than 15 days. Thus, the distribution of days from admission to the peak T. Bil level was significantly different between the two groups ($P = 0.0076$). It took a shorter time for the T. Bil level to reach the peak in AH-E than in AH-A.

## Discussion

No outbreaks of AH-E have been reported in Japan, and the incidence of documented sporadic cases of AH-E seems to be very low. Thus, Japan has been thought to be a nonendemic area for HEV, and AH-E has been considered as an imported hepatitis from endemic countries.[12,13] However, recent studies have revealed the presence of an indigenous strain of HEV in Japan, which had caused sporadic non-A, non-B, non-C hepatitis, and might develop to fulminant hepatitis.[22] Domestic animals such as swine may play an important role as reservoirs of HEV in Japan, as they do in other industrialized countries,[16] and now, AH-E is considered as a potential zoonosis in industrialized countries because of a close genetic relationship between swine and human

**Table 4.** Comparison of prognostic characteristics between AH-A and AH-E

| Characteristic | AH-A | AH-E | *P* |
|---|---|---|---|
| Mortality/Survival | 0/41 | 1/16 | 0.2931 |
| Period from the onset of illness to T. Bil <2.0mg/dl (days)[a] | | | |
| No. of patients | 34 | 15 | |
| Median (range) | 24.5 (12–55) | 30 (11–95) | 0.4036 |
| Period from the onset of illness to ALT <40IU/l (days) | | | |
| No. of patients | 41 | 16[b] | |
| Median (range) | 42 (16–66) | 36 (18–95) | 0.3421 |
| Days from admission to the peak T. Bil level[a] | | | 0.0076 |
| ≦7 | 4 | 8 | |
| 8–14 | 20 | 5 | |
| 15≦ | 10 | 2 | |

AH-A, acute hepatitis A; AH-E, acute hepatitis E, T. Bil; total bilirubin; ALT, alanine aminotransferase
[a]Excluding patients without jaundice on admission
[b]Excluding a patient who died of acute liver failure

HEV.[23] In the present study, we excluded patients with a history of traveling abroad within 3 months of onset of illness, to rule out imported AH-E, and we investigated AH-E caused by indigenous strains of HEV compared with AH-A. The clinical manifestations of AH-E in endemic areas are similar to those of AH-A. Both infections are transmitted by the fecal-oral route, and are related to poor hygiene and sanitation. Symptoms of AH-E and AH-A tend to be more severe in adults than in children.[24–26] However, a number of clinical and epidemiological differences between AH-E and AH-A have been revealed in recent studies. For example, HEV in endemic areas can cause severe fulminant hepatitis in pregnant women when the infection occurs during late pregnancy,[27] whereas exposure to HAV infection does not impose a high risk of fulminant hepatitis.[28,29] In a Japanese seroepidemiological study of anti-HEV IgG and anti-HAV in 1992 to 1993, the seroprevalence of anti-HAV was 65.3%–72.3%, but that of anti-HEV IgG was only 4.6%–6.7%.[30] Although the rate of positivity for anti-HAV increased with age in both males and females, the anti-HEV-positive rate tended to increase slightly with age in males, but not in females. Thus, HEV infection in Japan unlike HAV infection, was associated with male sex and older age.[30] Our data from the epidemiological study of acute hepatitis confirm that AH-E was more common in patients of older age and in men than AH-A in Japan. However, this result is different from that in endemic areas, where HEV infection was common in young adults age 15–40 years.[31–34]

In the present study, it was unclear when and why HEV infection occurred. However, among the patients who lived in Iwate, patients 8 and 9 were a retail meat dealer and a meat-processing trader, respectively. Patient 11 was a farmer working with pigs, and patient 12 had contact with pigs in his part-time job. In addition, patient 13 had raised young cattle, or cows for milk. The breeding of these domestic animals in farming families was principally undertaken by men over 40 years of age, whereas women and children rarely engaged in this work. These findings suggest that men are at a high risk for zoonotic HEV infection, particularly older men. Furthermore, of interest, is the finding that five HEV isolates; namely, HE-JA5, HE-JA6, HE-JA9, HE-JA11, and HE-JA23, were genetically close to the Japanese swine HEV isolate, swJ681, and an HEV isolate of HE-JA21 was also close to another Japanese swine HEV isolate, swJ570, based on the phylogenetic analysis of the ORF2 region.[16] In addition, it has been reported that the nucleotide identity of the ORF2 region (412nt) between HEV-Sendai and Japanese swine HEV isolate of swJ791 was 98.3%.[20] These results suggest that the infection sources of indigenous HEV strains in Japan are closely associated with domestic animals such as pigs.

Most of the HEV outbreaks in endemic areas of tropical and subtropical countries have been observed during the rainy season.[26] However, in industrialized countries such as Japan, it has been unclear whether the HEV infection occurred in a particular season. HAV infection was observed in winter or spring in Japan; however, no particular season was associated with HEV infection in this study. Therefore, we have to take into consideration HEV infection in the diagnosis of acute sporadic hepatitis in any season.

In this study, there were significant geographic differences in HEV genotypes in Japan. It was considered that genotype III was widespread principally on Honshu Island, whereas genotype IV was localized on Hokkaido Island. From the phylogenetic analysis of the ORF2 region, HE-JA3, HE-JA4, and HE-JA13 isolates of genotype IV closely resembled JKK-Sap (isolated from a patient who lived in Hokkaido) that has been

reported previously.[21] In genotype III, the nucleotide identity between HE-JA7 and HE-JA22, which were isolated at different times of onset in the same location of Iwate, was 100%. On the other hand, in genotype IV, HE-JA1, isolated from patient 1, who live in Hokkaido, shared 100% nucleotide identity with the HE-JI4 isolate, which was isolated in 2000 from a patient who lived in Tochigi, in Honshu island.[35] These findings suggest that in Japan, indigenous or native strains of HEV may be circulating in certain localized areas, but that some strains of HEV may be widespread with both types of strains causing AH-E.

Many symptoms of AH-E appeared to be the same as those of AH-A, but pyrexia of more than 38°C was observed more frequently in AH-A, and malaise was more frequent in AH-E. The reason for the more frequent observation of malaise in AH-E was thought to be that it was closely related to the age at onset of illness, and patients with AH-E were older than those with AH-A. But, why is pyrexia observed more frequently in AH-A than in AH-E? In this study, there were no specific physical findings in AH-E. However, there could be findings of a significant difference in the incidence of lymphadenopathy between AH-E and AH-A, if we were to study a larger number of cases of AH-E. On the other hand, our results showed several laboratory findings specific for AH-E compared with AH-A. The percentage of atypical lymphocytes, the ZTT value, and the IgM titer on admission were higher in AH-A patients than in AH-E patients, and the peak ALT level, peak ZTT value, and peak IgM titer were higher in AH-A patients than in AH-E patients. However, the T. Bil level was higher in AH-E than in AH-A, and more than 50% of patients with AH-E had reached the peak within 1 week of admission. These laboratory findings indicate that AH-A induces a strong immunological reaction compared with AH-E, whereas jaundice appears earlier in AH-E than in AH-A. Therefore, pyrexia may appear more frequently in AH-A as the result of the strong immunological reaction.

Although one patient with AH-E died of acute liver failure during the study period, there was no significant difference in the recovery period, according to the clinical course of T. Bil and ALT levels, between the AH-A and AH-E patients. It has been reported that risk factors that contribute to mortality in AH-A were older age, comorbid condition, and an underlying chronic liver disease.[36–38] On the other hand, risk factors for AH-E have not been clarified, except for older age and pregnancy. In experimental infections of nonhuman primates, the clinical presentation of hepatitis E is dose-dependent.[39] Thus, the severity of infection is directly related to the infectivity titer of challenge virus, and consistent demonstration of hepatitis in experimentally infected nonhuman primates has required challenge doses of at least 1000 times greater than the minimum dose required for infection.[40,41] It is not known whether such a clinical-to-infectious-dose relationship exists for naturally infected humans, but it is considered that the severity of AH-E depends on the infective viral load, based on the observed lower immunoreactivity in AH-E than in AH-A.

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# CASE REPORTS

## Infection of a Japanese Patient by Genotype 4 Hepatitis E Virus While Traveling in Vietnam

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**Cases of imported hepatitis E in industrialized countries infected with a genotype 1 hepatitis E virus (HEV) have been identified. We report a 56-year-old Japanese man who acquired infection with a genotype 4 HEV with 98.8% identity to a Vietnamese isolate after ingestion of uncooked shellfish while traveling in Vietnam.**

### CASE REPORT

A 56-year-old Japanese man visited an Internal Medicine Clinic in Tochigi, Japan, with complaints of dark urine and general malaise on 19 June 2003, 7 days after the onset of the illness. On that day, he was transferred to our hospital and was hospitalized with a clinical diagnosis of acute hepatitis. Physical examination on admission was essentially normal, except for jaundice. Laboratory data at admission showed an elevated total bilirubin level of 11.7 mg/dl, an aspartate aminotransferase level of 666 IU/liter, an alanine aminotransferase level of 972 IU/liter, an alkaline phosphatase level of 463 IU/liter, and a γ-glutamyl transpeptidase level of 258 IU/liter. The serum sample obtained at admission was negative for markers of hepatitis A, B, and C viruses. It was then tested for the immunoglobulin M (IgM) class of antibodies to hepatitis E virus (HEV) (anti-HEV IgM) by using an in-house enzyme immunoassay with purified recombinant open reading frame 2 (ORF2) protein that had been expressed in the pupae of silkworm (13) as the antigen probe, and it was also tested for HEV RNA by reverse transcription-PCR (RT-PCR) by a method described previously with primers targeting the ORF2 region (13). Based on positivity for anti-HEV IgM and HEV RNA, the patient was diagnosed as having sporadic acute hepatitis E. HEV RNA was detectable until the 12th hospital day. The maximum severity of the illness occurred at admission. After admission, he recovered rapidly and was discharged on the 13th hospital day.

The patient had no history of blood transfusion or liver disease and had no contact with pet animals or farm animals. Of note, he had traveled to Vietnam for sightseeing together with his wife and daughter from 21 April to 1 May 2003, and during their trip in Vietnam they consumed the same local Vietnamese cuisine or Western foods for every meal at restaurants. However, for lunch on 27 April 2003, the patient ingested only a raw shellfish (bivalve), i.e., a type of clam, which was served on the boat during a boat trip in Halong Bay, Vietnam. He developed hepatitis E 42 days after returning to Japan and 46 days after ingestion of the uncooked shellfish. His wife and daughter were negative for anti-HEV IgG and IgM and HEV RNA in the serum samples obtained on the 6th and 10th days of the patient's admission, respectively.

The presence of HEV RNA was confirmed by nested reverse transcription-PCR targeting a part of ORF1 (13). The amplified product of the ORF1 region and the amplified product of the ORF2 region from the serum sample that had been obtained at admission were sequenced directly on both strands. The HEV isolate (HE-JVN1) recovered from the infected patient was close to known human and swine genotype 4 HEV isolates, with 82.2 to 98.8% identity in a 326- or 412-nucleotide (nt) sequence of the ORF1 region (Table 1), and was most closely related to the V091 isolate (AB075967) of genotype 4 which had been isolated from a Vietnamese patient who contracted sporadic acute hepatitis E in Hanoi, Vietnam, in 2001, in a 326-nt sequence of the ORF1 region (7). In comparison with genotype 4 HEV strains reported from countries other than Vietnam, the HE-JVN1 isolate was only 87.4 to 89.8% similar to Chinese human isolates and 87.1 to 89.3% similar to Japanese human and swine isolates in the 412-nt ORF1 sequence. Upon comparison of a 241- to 412-nt sequence within ORF2, the HE-JVN1 isolate was closest to a Chinese swine HEV strain (SJ14 [AJ428856]), with 96.3% identity (20), and was only 83.4 to 94.0% similar to the remaining 78 human and swine isolates of Chinese, Indian, Indonesian, Japanese, or Taiwanese origin: no common ORF2 sequence of Vietnamese HEV isolates was available.

The phylogenetic tree constructed by the neighbor-joining method (15) based on the partial ORF1 sequence of 326 nt confirmed that the HE-JVN1 isolate belonged to genotype 4, and it segregated into a cluster consisting of six HEV strains, including V091, that had been isolated from six Vietnamese patients who had developed sporadic acute hepatitis E in Hanoi, Vietnam (Fig. 1).

HEV infections are endemic and frequently epidemic in

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TABLE 1. Comparison of the HEV isolate from the patient in the present study (HE-JVN1) with 104 human and swine HEV isolates of genotype 4 whose common 326- or 412-nt sequence in ORF1 or common 241- to 412-nt sequence in ORF2 is known

| Country | Host | ORF1 | | | ORF2 | | |
|---|---|---|---|---|---|---|---|
| | | No. of isolates compared | nt length compared | Identity (%) | No. of isolates compared | nt length compared | Identity (%) |
| China | Human | 2* | 412 | 87.4–89.8 | 22 | 301–412 | 83.4–94.0 |
| | Swine | 0 | –[a] | – | 5 | 300 | 85.3–96.3 |
| India | Swine | 0 | – | – | 12 | 241–263 | 85.4–88.7 |
| Indonesia | Swine | 0 | – | – | 1 | 412 | 90.8 |
| Japan | Human | 15 | 412 | 87.1–89.3 | 18 | 412 | 86.9–88.3 |
| | Swine | 2 | 412 | 88.1 | 10 | 412 | 87.1–87.9 |
| Taiwan | Human | 0 | – | – | 8 | 304–346 | 85.5–89.9 |
| | Swine | 0 | – | – | 3 | 304–346 | 87.2–90.5 |
| Vietnam | Human | 6 | 326 | 82.2–98.8 | 0 | – | – |

[a] –, Not applicable.

many developing countries in Asia, Africa, and Central America, where sanitation is suboptimal (14). Recent studies have documented that HEV-associated infection also occurs among individuals in industrialized countries with no history of travel to areas where HEV is endemic (6, 14, 16, 17). Although only one serotype has been recognized, extensive genomic diversity has been noted among HEV isolates, and HEV sequences have tentatively been classified into four genotypes (genotypes 1 to 4) (16). The majority of HEV infections are caused by genotype 1 in several developing countries in Asia and Africa, and one epidemic in Mexico caused by genotype 2 has been documented. Only isolated cases of infection with HEV of genotype 3 or 4 have been described in industrialized nations (16).

We encountered a patient with imported HEV who had traveled to Vietnam and ingested an uncooked shellfish and who was infected with an HEV strain of genotype 4 presumably indigenous to Vietnam, although many patients with imported HEV in industrialized countries are infected with a genotype 1 HEV which is prevalent in countries where the virus is hyperendemic (3, 8). In Japan, polyphyletic strains of HEV are

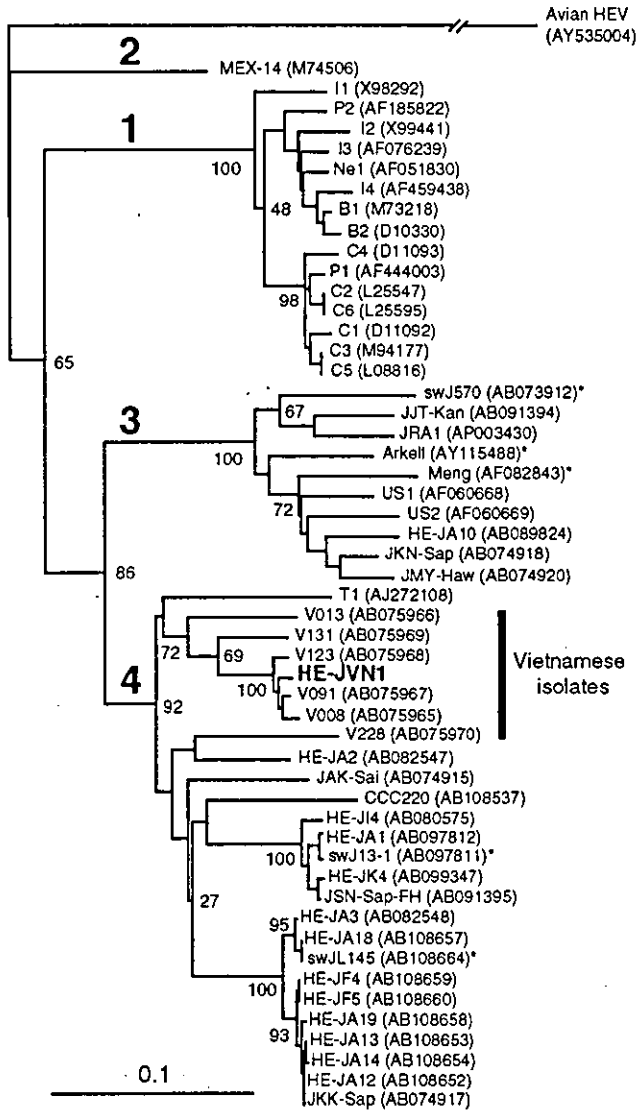


FIG. 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence (326 nt; nt 123 to 448 of the HE-JA10 genome, accession no. AB089824) of the ORF1 region of 52 human and swine HEV isolates using an avian HEV (AY535004) as an outgroup. In addition to 26 reported human and swine HEV isolates of genotypes 1 to 3 whose entire or nearly entire sequence is known, 26 reported isolates of genotype 4 whose common 326-nt sequence is available, as well as the HE-JVN1 isolate obtained in the present study, were included for comparison, with accession numbers in parentheses. The HE-JVN1 isolate is indicated by boldface type for visual clarity. The HEV strains of Vietnamese origin are shown by a vertical bar. The previously reported HEV sequences of genotype 1 are indicated with abbreviations in accordance with the recent review article by Schlauder and Mushahwar (16): B1 and B2 from Burma; C1, C2, C3, C4, C5, and C6 from China; I1, I2, I3, and I4 from India; Ne1 from Nepal; and P1 and P2 from Pakistan. Asterisks denote swine HEV strains. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings (4).

circulating and genotype 4 HEV strains have been recovered from patients with sporadic acute or fulminant hepatitis E who have no history of traveling abroad (13) and from farm pigs (18). Of interest, pairwise comparison and phylogenetic analysis of HEV sequences indicated that the HE-JVN1 strain isolated from the patient in the present study was most closely related to the HEV isolates of Vietnamese origin and not to those of Japanese origin, suggesting that he was infected with HEV while traveling in Vietnam. As reviewed by Smith (17), it is likely that foods can act as vehicles for transmission of HEV. Increasing evidence has indicated that hepatitis E is a zoonosis (6, 11, 12, 17), and that the zoonotic food-borne mode of transmission of HEV to humans, through ingestion of uncooked or undercooked liver from pigs (21), meat from a wild deer (19), or liver from a wild boar (9), seems to play an important role. Furthermore, the occurrence of acute hepatitis E in individuals after consumption of raw or uncooked shellfish has been reported (1, 10). In this regard, HEV is similar to hepatitis A virus, which is an important pathogen responsible for many food-borne outbreaks: the foods implicated in outbreaks of hepatitis A include shellfish (2, 5). The patient's spouse and daughter, who had traveled with the patient to Vietnam, did not eat raw shellfish and did not contract hepatitis. Although we could not prove that the shellfish consumed by our patient was the source of HEV infection, it appears likely that this was the case.Our study indicates that even an imported case of hepatitis E in Japan was infected with HEV of a genotype other than genotype 1, which is prevalent in areas where the virus is endemic; that consumption of uncooked shellfish contaminated with HEV may cause cryptic HEV infection; and that phylogenetic analysis of HEV strains may be useful for clinical surveys and for tracing infectious sources.**Nucleotide sequence accession numbers.** Nucleotide sequence data have been deposited in the GenBank/DDBJ/EMBL databases under accession nos. AB168095 (ORF1) and AB168096 (ORF2).

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# Prevalence of Antibodies to Hepatitis E Virus Among Japanese Blood Donors: Identification of Three Blood Donors Infected With a Genotype 3 Hepatitis E Virus

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Risk factors for acquiring hepatitis E among individuals in industrialized countries including Japan are not fully understood. We investigated whether Japanese blood donors with or without an elevated alanine aminotransferase (ALT) level are likely to have hepatitis E virus (HEV) infection. Serum samples were collected from 5,343 voluntary blood donors including 1,087 donors with elevated ALT of 61–966 IU/L and 4,256 donors with normal ALT (≤60 IU/L) at two Japanese Red Cross Blood Centers, and were tested for the presence of anti-HEV IgG by in-house enzyme-linked immunosorbent assay (ELISA). Overall, 200 donors (3.7%) were positive for anti-HEV IgG, including 32 (2.9%) with elevated ALT and 168 (3.9%) with normal ALT. Serum samples with anti-HEV IgG were further tested for the presence of anti-HEV IgM by in-house ELISA and for HEV RNA by reverse transcription (RT)-polymerase chain reaction (PCR). Three donors with ALT of 966, 62 or 61 IU/L were positive for anti-HEV IgM and HEV RNA. The HEV isolates obtained from the three viremic donors segregated into genotype 3, were 91.5–93.4% similar to each other in the 412 nucleotide sequence of open reading frame 2, and had the highest identity of 91.5–94.9% with the JRA1 isolate which was recovered from a Japanese patient with sporadic acute hepatitis E who had never been abroad, suggesting that these three HEV isolates are indigenous to Japan. This study suggests that a small but significant proportion of blood donors in Japan with or without elevated ALT are viremic and are potentially able to cause transfusion-associated hepatitis E. ***J. Med. Virol. 73:554–561, 2004.*** © 2004 Wiley-Liss, Inc.

**KEY WORDS:** hepatitis viruses; alanine aminotransferase; PCR; phylogenetic analysis

## INTRODUCTION

Hepatitis E is an important public health concern in many developing countries of Asia and Africa where sanitation is suboptimal, and it is also endemic in many industrialized countries including the United States, European countries, and Japan [Harrison, 1999; Purcell and Emerson, 2001; Smith, 2001; Okamoto et al., 2003]. The hepatitis E virus (HEV), the causative agent of hepatitis E, is an unclassified nonenveloped virus. Its genome is a single-stranded, positive-sense RNA of approximately 7.2 kb. It consists of a short 5′ untranslated region (UTR) followed by three partially overlapping open reading frames (ORFs: ORF1, ORF2, and ORF3), and then a short 3′UTR terminated by a poly(A) tract. ORF1 encodes viral non-structural proteins, ORF2 encodes the capsid protein, and ORF3 encodes a small phosphorylated protein [Reyes et al., 1990; Tam

The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB124818, AB154829, and AB154830.

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et al., 1991; Huang et al., 1992; Zafrullal et al., 1997; Wang et al., 1999, 2000]. Although only one serotype has been recognized, extensive genomic diversity has been noted among HEV isolates and HEV sequences have tentatively been classified into four genotypes (genotypes 1–4). The majority of HEV infections in developing countries are caused by genotype 1; one epidemic of infection with HEV of genotype 2 has been documented in Mexico; and only isolated cases of infection with HEV of genotype 3 or 4 have been described in industrialized nations [Schlauder and Mushahwar, 2001].

Transmission of HEV occurs primarily by the fecal-oral route through contaminated water supplies in developing countries. Recent studies have indicated that zoonosis is involved in the transmission of HEV, especially in industrialized countries where hepatitis E had been believed to be non-endemic [Meng et al., 1997, 1998; Harrison, 1999; Erker et al., 1999; Meng, 2000; Halbur et al., 2001; Okamoto et al., 2001; Smith, 2001; Nishizawa et al., 2003; Takahashi et al., 2003; Tei et al., 2003; Yazaki et al., 2003]. Numerous strains of HEV have been isolated from pigs in both developing and industrialized countries [Clayson et al., 1995; Chandler et al., 1999; Hsieh et al., 1999; Pina et al., 2000; Garkavenko et al., 2001; van der Poel et al., 2001; Arankalle et al., 2002; Huang et al., 2002; Pei and Yoo, 2002; Wu et al., 2002; Choi et al., 2003; Takahashi et al., 2003]. Increasing lines of evidence indicate that pigs are animal reservoirs for HEV and hepatitis E may be zoonotically transmitted from viremic animals to humans [Meng et al., 1997; Harrison, 1999; Meng, 2000; Smith, 2001]. In Japan, it has recently been reported that food-borne transmission of HEV may occur through ingestion of raw or undercooked meat including liver and intestine from infected swine, deer, or boar [Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003]. However, the modes of HEV transmission are still unclear for most patients with sporadic acute or fulminant hepatitis E in Japan [Takahashi et al., 2001, 2002a,b; Aikawa et al., 2002; Mizuo et al., 2002; Suzuki et al., 2002]. It has been reported that a substantial proportion of blood donors (3/200 or 1.5%) were positive for HEV RNA and viremic blood donors are potentially able to cause transfusion-associated hepatitis E in areas of high endemicity [Arankalle and Chobe, 1999, 2000]. Therefore, in the present study, we investigated whether Japanese blood donors with or without an elevated alanine aminotransferase (ALT) level are likely to have HEV infection in an attempt to gain insight into the possible blood-borne transmission of HEV in Japan which is now considered to be endemic.

## MATERIALS AND METHODS

### Serum Samples

Serum samples were collected from a total of 5,343 voluntary blood donors including 560 donors (age, 31.6 ± 10.5 [mean ± standard deviation, SD] years; 503 men and 57 women) with an elevated ALT level of 61–966 (range: 108 ± 59, mean ± SD) IU/L and 2,071 donors (38.3 ± 15.8 years; 1,030 men and 1,041 women) with a normal ALT level at the Japanese Red Cross Tochigi Blood Center (Center T) between April 2002 and June 2003, and 527 donors (37.0 ± 9.9 years; 480 men and 47 women) with an elevated ALT of 61–472 (96 ± 40) IU/L and 2,185 donors (39.1 ± 15.2 years; 1,121 men and 1,064 women) with a normal ALT at the Japanese Red Cross Yamaguchi Blood Center (Center Y) between May 2002 and October 2003. Blood Center T is located in a city in the northern part of mailand Honshu of Japan (Tochigi Prefecture) and Blood Center Y is located in a city in the southernmost part of mainland Honshu (Yamaguchi Prefecture). Additionally, periodic serum samples were obtained from three donors with transient HEV viremia. Stored serum samples that had been obtained from one donor were also used for retrospective analysis.

All 5,343 donors were negative for hepatitis B surface antigen, and antibodies to hepatitis C virus (HCV), human immunodeficiency virus (HIV) types 1 and 2, and human T-lymphotropic virus type 1, as well as for hepatitis B virus DNA, HCV RNA, and HIV type 1 RNA by the nucleic acid amplification test using Roche's Multiplex reagent [Mine et al., 2003].

### Detection of Antibodies to HEV

To detect anti-HEV IgG and anti-HEV IgM, enzyme-linked immunosorbent assay (ELISA) was carried out using purified recombinant ORF2 protein of HEV genotype 4 that had been expressed in the pupae of silkworm, as described previously [Mizuo et al., 2002]. The optical density (OD) of each sample was read at 450 nm. Using control sera from 200 healthy individuals (100 males and 100 females; age range: 16–24 years), the cut-off value was determined for the anti-HEV IgG assay as 0.180, and that for the anti-HEV IgM assay as 0.353 by the method described previously [Mizuo et al., 2002]. Samples with OD values for anti-HEV IgG or IgM equal to or greater than the respective cut-off value were considered to be positive for anti-HEV IgG or IgM, 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 or a mock protein obtained from the pupae of silkworm infected with nonrecombinant baculovirus. Briefly, when the OD value of the tested sample was less than 30% of the original value after absorption with the recombinant ORF2 protein and was greater than 70% of the original value after absorption with a mock protein, the sample was considered to be positive for anti-HEV.

### Detection of HEV RNA

Reverse transcription (RT)-polymerase chain reaction (PCR) was carried out for detection of HEV RNA in serum samples. Total RNA was extracted from 100 μl of serum, reverse transcribed, and then subjected to nested PCR with the ORF2 primers as described previously [Mizuo et al., 2002]. 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 was performed in duplicate, and reproducibility was confirmed. 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 [Mizuo et al., 2002].

### 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 12.2.0 (Genetyx Corp., Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ: National Institute of Genetics, Mishima, Japan) [Ina, 1994]. Sequence alignments were generated by CLUSTAL W (version 1.8) [Thompson et al., 1994]. A phylogenetic tree was constructed by the neighbor-joining method [Saitou and Nei, 1987] based on the partial nucleotide sequence of the ORF2 region (301 nucleotides [nt]). Bootstrap values were determined on 1,000 resamplings of the data sets [Felsenstein, 1985]. The final tree was obtained using the TreeView program (version 1.6.6) [Page, 1996].

### Statistical Analysis

Statistical analyses were performed using the $\chi^2$-test for comparison of proportions between two groups. Differences were considered to be statistically significant at $P < 0.05$.

## RESULTS

### Prevalence of Anti-HEV IgG Among Voluntary Blood Donors in Two Distinct Geographic Regions in Japan

A total of 5,343 serum samples obtained from apparently healthy blood donors at two Red Cross Blood Centers (T and Y), located in cities in the northern part and southernmost part, respectively, of Honshu Island of Japan, were tested for the presence of anti-HEV IgG. At Blood Center T, anti-HEV IgG was detected in 5.5% (144/2,631) of the tested population; it was detected in 4.1% of the 560 donors with elevated ALT of 61–966 IU/L and in 5.8% of the 2,071 donors with normal ALT. On the other hand, the detection rate of anti-HEV IgG among the 2,712 donors at Blood Center Y was 2.1%, which was significantly lower than that at Blood Center T ($P < 0.0001$). Anti-HEV IgG was detected in 1.7% in the 527 donors with an elevated ALT level of 61–472 IU/L ($P = 0.0193$ in comparison with the respective parameter in Center T), and in 2.2% of the 2,185 donors with normal ALT ($P < 0.0001$) (Table I). The age-dependent prevalence of anti-HEV IgG was compared among blood donors in relation to gender and ALT at the two blood centers (Table I). The prevalence of anti-HEV IgG increased with age among both the male and female

TABLE I. Age-Specific Prevalence of Anti-HEV IgG Among Apparently Healthy Individuals With or Without an Elevated ALT at Two Blood Centers in Japan

| | Blood center T | | | | Blood center Y | | | |
|---|---|---|---|---|---|---|---|---|
| | ALT ≤60 IU/L | | ALT >61 IU/L | | ALT ≤60 IU/L | | ALT >61 IU/L | |
| Age (year) | Male | Female | Male | Female | Male | Female | Male | Female |
| 16–19 | 2/185 (1.1%) | 1/173 (0.6%) | 2/91 (2.2%) | 0/16 | 1/157 (0.6%) | 1/173 (0.6%) | 0/13 | 0/4 |
| 20–29 | 7/195 (3.6%) | 3/208 (1.4%) | 2/113 (1.8%) | 0/13 | 5/209 (2.4%) | 2/200 (1.0%) | 0/99 | 0/6 |
| 30–39 | 7/175 (4.0%) | 5/189 (2.6%) | 8/183 (4.4%) | 0/13 | 3/196 (1.5%) | 2/180 (1.1%) | 3/196 (1.5%) | 0/14 |
| 40–49 | 19/175 (10.9%) | 15/156 (9.6%) | 6/89 (6.7%) | 0/10 | 6/212 (2.8%) | 2/194 (1.0%) | 5/120 (4.2%) | 0/10 |
| 50–59 | 23/152 (15.1%)[a] | 10/157 (6.4%)[a] | 4/24 (16.7%) | 0/4 | 9/183 (4.9%) | 7/168 (4.2%) | 1/45 (2.2%) | 0/11 |
| 60–69 | 18/148 (12.2%) | 11/158 (7.0%) | 1/3 (33.3%) | 0/1 | 6/164 (3.7%) | 3/149 (2.0%) | 0/7 | 0/2 |
| Subtotal | 76/1,030 (7.4%)[b,d] | 45/1,041 (4.3%)[b,e] | 23/503 (4.6%)[f,h] | 0/57[f] | 30/1,121 (2.7%)[c,d] | 17/1,064 (1.6%)[c,e] | 9/480 (1.9%)[g,h] | 0/47[g] |
| Total | 121/2,071 (5.8%)[i,k] | | 23/560 (4.1%)[j,l] | | 47/2,185 (2.2%)[j,k] | | 9/527 (1.7%)[j,l] | |
| Grand total | 144/2,631 (5.5%)[m] | | | | 56/2,712 (2.1%)[m] | | | |

[a] $P = 0.0127$.
[b] $P = 0.0024$.
[c] $P = 0.0824$ (not significant [NS]).
[d] $P < 0.0001$.
[e] $P = 0.0002$.
[f] $P = 0.1068$ (NS).
[g] $P = 0.3437$ (NS).
[h] $P = 0.0247$.
[i] $P = 0.0717$ (NS).
[j] $P = 0.5207$ (NS).
[k] $P < 0.0001$.
[l] $P = 0.0193$.
[m] $P < 0.0001$.

donors at the two blood centers regardless of the ALT level, although none of the female donors with elevated ALT tested positive for anti-HEV IgG at both blood centers, probably due to the small number of female donors tested in all age groups. At Blood Center T, the prevalence of anti-HEV IgG was significantly higher among donors with normal ALT aged ≥40 years than among those aged <40 years in the males (12.6% vs. 2.9%, $P < 0.0001$) and females (7.6% vs. 1.6%, $P < 0.0001$), and among male donors with elevated ALT aged ≥40 years than among those aged <40 years (9.5% vs. 3.1%, $P = 0.0039$). Similar age-specific differences in the prevalence of anti-HEV IgG were observed at Blood Center Y, as indicated in Table I. Upon comparison of donors with normal ALT at Blood Center T, HEV infection was significantly more frequent among the male donors than among the female donors (7.4% vs. 4.3%, $P = 0.0024$). The prevalence of anti-HEV IgG was also higher among the male donors with normal ALT than among the female donors with normal ALT at Blood Center Y and higher among the male donors with elevated ALT than among female donors with elevated ALT at both blood centers.

### Prevalence of Anti-HEV and HEV RNA Among Blood Donors in the Two Blood Centers in Japan, Stratified by ALT Level

Twenty-one donors with anti-HEV IgG of high $OD_{450}$ value (>1.000) were found at Blood Center T, including one (50%) with an ALT of >500 IU/L, two (1.0%) with an ALT of 101–200 IU/L, seven (2.0%) with an ALT of 61–100 IU/L, and 11 (0.5%) with normal ALT (Table II). At Blood Center Y, only seven donors (0.3%) with anti-HEV IgG of high $OD_{450}$ value (>1.000) were found, in which all seven donors had a normal ALT. The 200 donors who were positive for anti-HEV IgG were tested for the presence of anti-HEV IgM. No donor at Blood Center Y was seropositive for anti-HEV IgM, regardless of the ALT level. In contrast, at Blood Center T, three donors (3/560 or 0.5%) with an elevated ALT level of 61, 62, or 966 IU/L were positive for anti-HEV IgM, all of whom had anti-HEV IgG of high $OD_{450}$ value (>1.000), and all 121 donors with normal ALT who were positive for anti-HEV IgG, were negative for anti-HEV IgM. Two of the three donors with anti-HEV IgG and anti-HEV IgM had detectable HEV RNA. No other donor with anti-HEV IgG of high $OD_{450}$ value (>1.000) was positive for HEV RNA.

### Detection of Anti-HEV and HEV RNA in Periodic Serum Samples Obtained From Three Donors With Anti-HEV IgM

Periodic serum samples were obtained from the three donors (Donors 1–3) with elevated ALT who had been found to be positive for anti-HEV IgM in the above-mentioned screening test after informed consent, and tested for anti-HEV IgG, anti-HEV IgM, and HEV RNA (Table III). None of the three donors had signs or symptoms of HEV infection during the observation period. Donor 1 was a 54-year-old male with a history of >100 donations. His blood screening tests including liver function tests were exclusively normal at the previous donations. On the initial sampling day of the present study (September 12, 2002), his liver enzyme levels were markedly elevated (ALT, 966 IU/L; AST, 815 IU/L), he was positive for both anti-HEV IgG and anti-HEV IgM, and HEV RNA was detectable. Thereafter, his liver enzyme levels were within the normal range and HEV RNA was undetectable. The anti-HEV IgG level continued to be high until the end of the observation period (November 26, 2003), at which time the $OD_{450}$ value was 2.632. The relative titer of IgM antibody was highest on day 61 ($OD_{450}$ value, 2.279) and then gradually decreased, and it continued to be positive

TABLE II. Prevalence of Anti-HEV and HEV RNA Among Blood Donors at Two Distinct Blood Centers in Japan, Stratified by ALT Level

| ALT level (IU/L) | No. of donors tested | No. of donors with anti-HEV IgG ($OD_{450}$ value) of | | No. of donors with anti-HEV IgM[b] | No. of donors with HEV RNA[c] |
|---|---|---|---|---|---|
| | | ≥0.180[a] | >1.000 | | |
| Blood center T | | | | | |
| >500 | 2 | 1 (50%) | 1 (50%) | 1 (50%) | 1 (50%) |
| 201–500 | 21 | 0 | 0 | 0 | 0 |
| 101–200 | 193 | 5 (2.6%) | 2 (1.0%) | 0 | 0 |
| 61–100 | 344 | 17 (4.9%) | 7 (2.0%) | 2 (0.6%) | 1 (0.3%) |
| Total | 560 | 23 (4.1%) | 10 (1.8%) | 3 (0.5%) | 2 (0.4%) |
| 5–60 | 2,071 | 121 (5.8%) | 11 (0.5%) | 0 | 0 |
| Blood center Y | | | | | |
| >500 | 0 | 0 | 0 | 0 | 0 |
| 201–500 | 13 | 0 | 0 | 0 | 0 |
| 101–200 | 149 | 0 | 0 | 0 | 0 |
| 61–100 | 365 | 9 (2.5%) | 0 | 0 | 0 |
| Total | 527 | 9 (1.7%) | 0 | 0 | 0 |
| 5–60 | 2,185 | 47 (2.2%) | 7 (0.3%) | 0 | 0 |

[a]Cut-off value for anti-HEV IgG was 0.180.
[b]Positivity for anti-HEV IgM was tested in blood donors with anti-HEV IgG.
[c]Positivity for HEV RNA was tested in blood donors with anti-HEV IgG of >1.000.

TABLE III. Laboratory Parameters, Anti-HEV Antibody Levels and HEV RNA in Periodic Serum Samples Obtained From Three Blood Donors With Transient HEV Viremia at Blood Center T

| Blood donor | Date of sampling | ALT (IU/L) | AST (IU/L) | $OD_{450}$ value in anti-HEV assay[a] | | HEV RNA |
|---|---|---|---|---|---|---|
| | | | | IgM-class | IgG-class | |
| 1 | **2002/9/12**[b] | **966** | **815** | **0.940** (+) | **>3.000** (+) | + |
| | 2002/10/27 | 25 | 26 | 2.020 (+) | >3.000 (+) | − |
| | 2002/11/12 | 20 | 20 | 2.279 (+) | >3.000 (+) | − |
| | 2002/12/15 | 22 | 24 | 1.127 (+) | >3.000 (+) | − |
| | 2003/1/29 | 17 | 20 | 0.937 (+) | >3.000 (+) | − |
| | 2003/2/16 | 19 | 24 | 0.838 (+) | >3.000 (+) | − |
| | 2003/3/8 | 16 | 21 | 0.732 (+) | >3.000 (+) | − |
| | 2003/4/6 | 24 | 27 | 0.676 (+) | >3.000 (+) | − |
| | 2003/6/28 | 12 | 18 | 0.571 (+) | >3.000 (+) | − |
| | 2003/7/29 | 18 | 25 | 0.460 (+) | 2.684 (+) | − |
| | 2003/9/1 | 14 | 21 | 0.302 (−) | 2.592 (+) | − |
| | 2003/11/11 | 14 | 20 | 0.217 (−) | 2.362 (+) | − |
| | 2003/11/26 | 12 | 22 | 0.255 (−) | 2.632 (+) | − |
| 2 | **2002/4/22**[b] | **61** | **22** | **>3.000** (+) | **>3.000** (+) | + |
| | 2002/8/14 | 19 | 16 | 0.390 (+) | >3.000 (+) | − |
| | 2003/8/13 | 20 | 15 | 0.135 (−) | 2.181 (+) | − |
| 3 | 2002/11/14 | 49 | 51 | 0.025 (−) | 0.008 (−) | − |
| | 2003/3/10 | 68 | 35 | 0.030 (−) | 0.014 (−) | + |
| | **2003/6/10**[b] | **62** | **37** | **0.792** (+) | **2.611** (+) | − |

[a]Cut-off values for anti-HEV IgM and IgG were 0.353 and 0.180, respectively.
[b]Index sample that was first found to be positive for anti-HEV IgM and IgG is indicated in boldtype.

until day 320 after the initial testing. Donor 2 was a 35-year-old male with normal liver enzyme levels (ALT, 24 IU/L; AST, 16 IU/L) at the time of his previous donation 126 days before the initial sampling day of April 22, 2002. On the first sampling day, the ALT was slightly elevated (61 IU/L), but the AST was within the normal range (22 IU/L). He was highly positive for anti-HEV IgG and anti-HEV IgM, and HEV RNA was detectable on the initial sampling day. After 16 months, he remained positive for anti-HEV IgG antibody with an $OD_{450}$ value of 2.181, but he was negative for IgM antibody. Donor 3 was a 41-year-old male. On the initial testing, he had a slightly elevated ALT level of 62 IU/L and was positive for both anti-HEV IgG and anti-HEV IgM, but HEV RNA was undetectable. Two stored serum samples that had been obtained 92 and 208 days before the initial testing, were tested for the presence of anti-HEV IgG, anti-HEV IgM, and HEV RNA. Although the two serum samples were negative for both anti-HEV antibodies, the serum sample obtained 3 months before the initial testing was repeatedly positive for HEV RNA and the donor had an elevated ALT level of 68 IU/L at that time. Fortunately, this blood was not utilized for transfusion due to an abnormal ALT level. Consequently, three blood donors with anti-HEV IgG and anti-HEV IgM were found to be transiently viremic.

### Genetic Analysis of HEV Isolates Recovered From Three Viremic Donors

The three HEV isolates recovered from the transiently viremic donors (Donors 1–3) were named HE-JBD1, HE-JBD2, and HE-JBD3, respectively. The 412 nt sequence of ORF2 of these HEV isolates were determined and compared with each other and with that of known human and swine HEV isolates of genotypes 1–4. These three HEV isolates were 91.5–93.4% similar to each other, and were most closely related to the prototype Japanese isolate of genotype 3 (JRA1 [accession no. AP003430]) with nucleotide sequence identity of 91.5–94.9%, and were only 78.3–79.6%, 74.3–77.4%, and 78.8–79.9% similar to the B1 isolate (M73218) of genotype 1, MEX-14 isolate (M74506) of genotype 2, and T1 isolate (AJ272108) of genotype 4, respectively. The phylogenetic tree constructed based on the common 301 nucleotides within the ORF2 sequence confirmed that the HE-JBD1, HE-JBD2, and HE-JBD3 isolates obtained in the present study belonged to genotype 3, and that they segregated into the cluster consisting of Japanese HEV strains of the same genotype that had been recovered from humans (JRA1, HE-JO-1982, HE-JA5, HE-JA6, HE-JA9, HE-JA11, HE-JA21, HE-JA23, and HE-JF2) and swine (swJ570 and swJ681), supporting the indigenous nature of these three blood donor isolates.

## DISCUSSION

HEV is a significant cause of epidemic and sporadic acute viral hepatitis in developing countries of Asia and Africa, and HEV-associated hepatitis also occurs sporadically in some industrialized countries including the United States, European countries, and Japan. Transmission of HEV occurs primarily by the fecal-oral route through contaminated water supplies in many developing countries. However, in industrialized countries where sanitation and hygiene are well established, the chance of fecal-oral transmission of HEV may be negligible, and the risk factors for acquiring hepatitis

E among individuals in industrialized countries are not fully understood [Harrison, 1999; Meng, 2000; Purcell and Emerson, 2001; Smith, 2001; Emerson and Purcell, 2003; Okamoto et al., 2003]. As reviewed by Smith [2001], it is likely that several forms of HEV transmission other than fecal-oral transmission occur in industrialized countries with no or low HEV endemicity: (a) zoonotic infection, (b) transmission by food, and (c) transmission via blood transfusion. Accumulated evidence indicates that pigs are animal reservoirs for HEV and hepatitis E may be zoonotically transmitted from viremic animals to humans [Meng et al., 1997, 1998; Erker et al., 1999; Harrison, 1999; Meng, 2000; Pina et al., 2000; Halbur et al., 2001; Smith, 2001; Wang et al., 2002]. In Japan where hepatitis E had been believed to be non-endemic but is now considered to be endemic [Okamoto et al., 2001; Takahashi et al., 2001, 2002a,b, 2003; Mizuo et al., 2002; Nishizawa et al., 2003], evidence for food-borne transmission of HEV through the consumption of HEV-contaminated food has been accumulating; HEV contamination was found in various foods including raw pig livers that are available in grocery stores in Hokkaido, which is located in the northern part of Japan and where hepatitis E is most prevalent in Japan [Yazaki et al., 2003]; and raw meat from a wild deer in Hyogo Prefecture which is located in the southern part of mainland Honshu [Tei et al., 2003]. Japanese people have a peculiar habit of eating uncooked seafood (sushi or sashimi), and less frequently, raw or undercooked meat including liver and intestine from pigs and other animals. These eating habits may explain, at least partly, the cryptic endemicity of HEV in Japan. However, the source and route of HEV transmission remain unclear for most cases of sporadic acute or fulminant hepatitis E in areas other than the above-mentioned areas of Japan [Takahashi et al., 2001, 2002a,b; Aikawa et al., 2002; Mizuo et al., 2002; Suzuki et al., 2002; Tokita et al., 2003].

In the present study, in an attempt to gain insight into the possible blood-borne transmission of HEV in Japan, we investigated the prevalence of anti-HEV IgG among voluntary blood donors who donated blood in two distinct geographic regions that are located in the northern and southernmost parts of mainland Honshu (Blood Center T in Tochigi Prefecture and Blood Center Y in Yamaguchi Prefecture, respectively). At least two cases of sporadic acute hepatitis E with unknown transmission mode(s) have been recognized thus far in Tochigi Prefecture [Takahashi et al., 2002b; Kuno et al., 2003], although no case has been reported in Yamaguchi Prefecture, reflecting the observed higher prevalence of anti-HEV IgG among blood donors at Blood Center T (5.5% vs. 2.1%, $P < 0.0001$). The prevalence of anti-HEV IgG was clearly associated with age among the blood donors at both blood centers, corroborating the previous report on the prevalence of anti-HEV IgG antibody among 900 Japanese patients in that the anti-HEV prevalence of anti-HEV among patients over 30 years of age was associated with age and increased in a cumulative fashion in all three geographical regions (northern, central, and southern prefectures of Japan) [Li et al., 2000]. HEV infection was associated with male sex in all age groups studied in at the two blood centers in the present study, consistent with the observed higher prevalence of clinical HEV infection among male patients in Japan [Mizuo et al., 2002].

In the current study, three viremic blood donors who all had an elevated ALT, were identified among the donors with a high level of anti-HEV IgG (>1.000) at Blood Center T which is located in a region where the prevalence of anti-HEV IgG is high, although none of the donors with normal ALT and a high level of anti-HEV IgG had detectable HEV RNA. In contrast, at Blood Center Y which is located in a region where HEV infection is less prevalent, none of the blood donors who had a high level of anti-HEV IgG with or without an elevated ALT were positive for HEV RNA. These results suggest that a proportion of blood donors in areas where HEV infection is prevalent in Japan are viremic and are potentially able to cause transfusion-associated hepatitis E, as has been reported in India which has high endemicity of HEV; in two studies of Arankalle and Chobe [1999, 2000], three (1.5%) of 200 voluntary blood donors were positive for HEV RNA. As the three viremic donors identified in the present study had an elevated ALT, the blood from the three donors were not used for transfusion. As the majority of donors with elevated ALT and the donors with normal ALT were not tested for HEV viremia in the present study, we cannot conclude that the serum ALT level could be a surrogate marker for exclusion of blood donors with ongoing HEV infection. Based on the current study, however, we would like to consider that ALT testing is useful for exclusion of donors with HEV viremia, at least partially, with the aim of preventing transfusion-associated hepatitis E. As two of the three infected donors had only a slightly elevated ALT level of 61 or 62 IU/L, it seems likely that even donors with a normal ALT level (≤60 IU/L) may have detectable HEV RNA, although the proportion of such donors may be significantly small. In support of this speculation, it has been reported in a newspaper (ABC Newsletter January 24, 2003; accessible at http://www.americasblood.org) that a Japanese man in his 60s contracted hepatitis E from a blood transfusion that he had received during heart surgery at a city hospital in Hokkaido in 2002, where hepatitis E is most prevalent in Japan as described above.

Multiple HEV strains of genotype 3 or 4 have been isolated from Japanese patients with sporadic acute or fulminant hepatitis E as well as from farm pigs in Japan [Okamoto et al., 2003]. Reflecting the polyphyletic nature of human and swine HEV isolates of Japan origin, the HEV isolates recovered from three viremic donors in the present study, differed by 6.6–8.5% from each other, although they belonged to the same genotype (genotype 3) with the highest nucleotide sequence identity of 91.5–94.9% with the JRA1 isolate that is believed to be indigenous to Japan [Takahashi et al., 2001]. Three distinct swine HEV strains (swJ570, swJ681, and swJ791 in Fig. 1) of genotype 3 [Okamoto

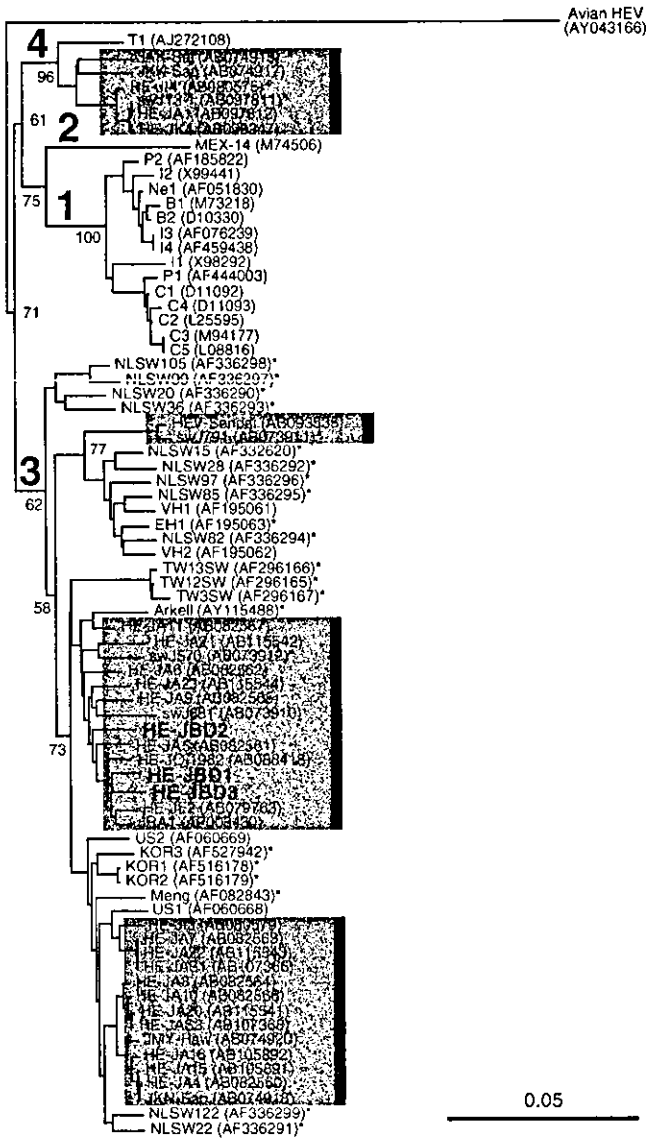


Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2 region (301 nt) of 75 HEV isolates, using a chicken HEV (AY043166) as an outgroup. In addition to the HE-JBD1, HE-JBD2, and HE-JBD3 isolates found in the present study which are indicated in bold type, 72 reported HEV isolates of genotypes 1–4 whose common 301 nt sequence is known are included for comparison and their accession nos. are shown in parentheses. The previously reported HEV sequences of genotype 1 are indicated with abbreviations in accordance with the recent review article by Schlauder and Mushahwar [2001]: B1 and B2 in Burma; C1, C2, C3, C4, and C5 in China; I1, I2, I3 and I4 in India; Ne1 in Nepal; and P1 and P2 in Pakistan. Asterisks denote swine HEV strains. The human and swine HEV isolates of Japan origin are shaded and indicated with vertical bars for visual clarity. Bootstrap values are indicated for the major nodes as a percentage obtained from 1,000 resamplings of the data.

et al., 2001] and three different human HEV strains (HE-JI3, HE-JI4, and HE-JK4 in Fig. 1) of genotype 3 or 4 [Kuno et al., 2003; Takahashi et al., 2002b] have been isolated in the same prefecture as that of the three viremic donors, and they all share only up to 90.7% identity with the HE-JBD1, HE-JBD2, and HE-JBD3 isolates obtained from the viremic donors 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.

In conclusion, three blood donors with HEV viremia were identified among voluntary blood donors with an elevated ALT at a blood center located in the northern part of mainland Honshu of Japan where HEV infection is prevalent. Our study supports the possibility of transfusion-associated hepatitis E in countries like Japan where HEV is circulating and where there are symptom-free HEV RNA-positive donors. A much larger study with a greater number of blood donors with or without an elevated ALT level is needed to assess the frequency of HEV-viremic blood donors and the usefulness of ALT testing for excluding viremic donors, although the major contributing factors to an elevated ALT level in blood donors are known to be alcohol consumption and obesity, and to elucidate whether or not a screening test for HEV infection is required for blood donors, taking into consideration the geographic region in Japan.

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