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Prevalence of Hepatitis E Virus Infection Among Hemodialysis Patients in Japan: Evidence for Infection With a Genotype 3 HEV by Blood Transfusion

Takehiro Mitsui,[1] Yukie Tsukamoto,[1] Chikao Yamazaki,[1] Kazuo Masuko,[1] Fumio Tsuda,[2] Masaharu Takahashi,[3] Tsutomu Nishizawa,[3] and Hiroaki Okamoto[3]*

[1]*Masuko Memorial Hospital and Masuko Institute for Medical Research, Aichi-Ken, Japan*
[2]*Department of Medical Sciences, Toshiba General Hospital, Tokyo, Japan*
[3]*Division of Virology, Department of Infection and Immunity, Jichi Medical School, Tochigi-Ken, Japan*

To investigate the prevalence of hepatitis E virus (HEV) infection among patients on maintenance hemodialysis, serum samples collected in January 2003 from 416 patients who had been undergoing hemodialysis for 7.6 ± 6.3 (mean ± standard deviation) (range, 0.3–26.0) years in a dialysis unit in Japan and serum samples that had been collected from these patients at the start of hemodialysis were tested for IgG antibodies to HEV (anti-HEV IgG) by an "in-house" enzyme-linked immunosorbent assay (ELISA). Overall, 39 patients (9.4%) had anti-HEV IgG in January 2003, and included 35 patients (8.4%) who had already been positive for anti-HEV IgG at the start of hemodialysis and 4 patients (1%) who seroconverted after initiation of hemodialysis. Periodic serum samples that had been collected from the four seroconverted patients were tested for HEV antibodies and HEV RNA. The four patients became positive for anti-HEV IgG in 1979, 1980, 1988, or 2003, and continued to be seropositive until the end of the observation period. Although anti-HEV IgM was not detectable in the four patients, three were infected transiently with apparently Japanese indigenous HEV strains of genotype 3. The patient who contracted HEV infection in 1979 had been transfused with 2 U of blood 21 days before the transient viremia: one of the two stored pilot serum samples had detectable HEV RNA with 100% identity to that recovered from the patient. Our study provides evidence of transfusion-transmitted HEV infection in Japan in 1979, and that the prevalence of de novo HEV infection during hemodialysis was low (1.1% or 4/374). ***J. Med. Virol. 74:563–572, 2004.***
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KEY WORDS: hepatitis viruses; genotype; PCR; phylogenetic analysis; route of transmission

INTRODUCTION

Hepatitis E, which is caused by hepatitis E virus (HEV), is an important public health concern in many developing countries where sanitation is suboptimal: large epidemics of hepatitis E have been reported in Asia, Africa, and Latin America [Purcell and Emerson, 2001]. Although only sporadic cases of acute hepatitis E have been reported in many industrialized countries including the United States, European countries, and Japan [Harrison, 1999; Purcell and Emerson, 2001; Schlauder and Mushahwar, 2001; Smith, 2001; Okamoto et al., 2003], a significant proportion of healthy individuals in industrialized countries are seropositive for HEV antibodies [Mast et al., 1997; Thomas et al., 1997]. HEV was recently classified as the sole member of the genus *Hepevirus* in the family Hepeviridae. Its

The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB175483–AB175486.

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*Correspondence to: Dr. Hiroaki Okamoto, Division of Virology, Department of Infection and Immunity, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-Machi, Tochigi-Ken 329-0498, Japan. E-mail: hokamoto@jichi.ac.jp

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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 [Reyes et al., 1990; Tam et al., 1991; Huang et al., 1992; Wang et al., 2000].

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]. Recent studies have indicated that hepatitis E is a zoonosis [Meng et al., 1997, 1998; Erker et al., 1999; Harrison, 1999; Meng, 2000; Halbur et al., 2001; Okamoto et al., 2001; Smith, 2001; Nishizawa et al., 2003; Tei et al., 2003; Yazaki et al., 2003]. Numerous strains of HEV of genotype 3 or 4 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; Wang et al., 2002; Wu et al., 2002; Choi et al., 2003; Takahashi et al., 2003].

In Japan, it has been shown that the circulating HEV strains are polyphyletic [Mizuo et al., 2002] and that the zoonotic food-borne mode of transmission of HEV to humans may play an important role in the occurrence of hepatitis E [Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Tamada et al., 2004]. However, the mode of HEV transmission was not clear in the majority of 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]. Recently, a patient who was infected with HEV via transfused blood from a voluntary blood donor was reported, and the authors stated that the potential risk of post-transfusion hepatitis E should be considered even in non- or low-endemic countries including Japan [Matsubayashi et al., 2004]. The majority of patients on maintenance hemodialysis have a history of blood transfusion. Some investigators [Halfon et al., 1994; Ding et al., 2003] observed a high prevalence of anti-HEV antibody among their hemodialysis patients. However, other investigators found only a few anti-HEV-positive patients among their hemodialysis populations [Courtney et al., 1994; Psichogiou et al., 1996; Fabrizi et al., 1997].

Therefore, in the present study, we determined the prevalence of HEV infection among 416 patients undergoing maintenance hemodialysis at a single dialysis unit in Japan using serum samples that had been obtained at the start of hemodialysis and serum samples that had been periodically collected from each patient and stored since the initiation of hemodialysis, to investigate whether hemodialysis and blood transfusion are associated with increased risk of HEV infection. Furthermore, stored pilot serum samples of transfused blood were tested for the presence of HEV RNA to clarify whether a hemodialysis patient who contracted de novo HEV infection 20 days after blood transfusion acquired transfusion-associated HEV infection.

MATERIALS AND METHODS

Serum Samples

Serum samples were collected in January 2003 from a total of 416 hemodialysis patients (age, 60.1 ± 12.6 [mean ± standard deviation, SD] years; 274 men and 142 women) who had been receiving maintenance hemodialysis in the dialysis unit of Masuko Memorial Hospital in Nagoya, Japan, for more than 3 months (7.6 ± 6.3 [range, 0.3–26.0] years). Additionally, stored serum samples that had been obtained from the 416 patients at the start of hemodialysis were used. From the four patients who became seropositive for HEV infection after the initiation of hemodialysis, stored serum samples that had been obtained periodically (semimonthly between 1977 and 2001 and monthly thereafter) were also used in this retrospective analysis. This study conforms to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the ethics committee at the institution. Informed consent was obtained from each patient.

Detection of Antibodies to HEV

The serum samples were tested for the IgG, IgM, and IgA classes of anti-HEV by in-house enzyme-linked immunosorbent assay (ELISA), 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; Tokita et al., 2003]. The optical density (OD) of each sample was read at 450 nm. The cut-off value used for the anti-HEV IgG assay was 0.152 that for the anti-HEV IgM assay was 0.353, and that for the anti-HEV IgA assay was 0.350. 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 or a mock protein obtained from the pupae of silkworm infected with non-recombinant baculovirus. Briefly, if the OD value of the tested sample was less than 30% of the original value after absorption with the recombinant ORF2 protein and if it was greater than 70% of the original value after absorption with the mock protein, the sample was considered to be positive for anti-HEV. The serum samples were also tested for anti-HEV IgM using a commercially available kit (Genelabs Diagnostics, Pte. Ltd., Singapore Science Park, Singapore).

Detection of HEV RNA

Reverse transcription (RT)-polymerase chain reaction (PCR) was carried out for detection of HEV RNA in

the serum samples. Total RNA was extracted from 100 μl of serum, reverse transcribed, and then subjected to nested PCR with primers targeting the ORF2 region 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. To avoid contamination during PCR procedures, the guidelines of Kwok and Higuchi [1989] were strictly observed. 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].

Serological Tests for Other Viral Infections

Serum samples were tested for hepatitis B surface antigen (HBsAg) and the corresponding antibody (anti-HBs) by passive hemagglutination using commercial assay kits (Mycell II HBsAg and Mycell II anti-HBs, respectively; Institute of Immunology, Co. Ltd., Tokyo, Japan). The presence of antibody to hepatitis B core antigen (anti-HBc) was determined by hemagglutination inhibition by the method described previously [Iizuka et al., 1992]. The presence of antibody to hepatitis C virus (HCV) (anti-HCV) was determined by a commercially available enzyme immunoassay kit (HCV · EIA II, Abbott Japan, Tokyo, Japan).

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 datasets [Felsenstein, 1985]. The final tree was obtained using the TreeView program (version 1.6.6) [Page, 1996].

Statistical Analysis

Data are presented as the mean ± SD. Statistical analyzes were carried out using the Welch's t-test for comparison of continuous variables between two groups, and the χ^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, HBsAg, and Antibodies to HBV and HCV Among Hemodialysis Patients

Serum samples obtained from 416 patients on maintenance hemodialysis at a dialysis unit of a city hospital in Japan in January 2003, were tested for the presence of anti-HEV IgG. Anti-HEV IgG was detected in 39 (9.4%) of the 416 patients, with a higher prevalence among males than among females (11.3% vs. 5.6%) (Table I). The prevalence of anti-HEV IgG tended to be lower among patients aged <40 years than among those aged ≥40 years, but the difference fell short of being statistically significant. There were nine patients with anti-HEV IgG of high OD_{450} value (≥1.000), including two (5.0%) in the age group of 40–49 years, four (3.7%) in the age group of 50–59 years, two (1.4%) in the age group of 60–69 years, and one (1.3%) in the age group of 70–79 years. Of note, two patients had anti-HEV IgG of very high OD_{450} value (≥3.000), but they were negative for both anti-HEV IgM and HEV RNA.

Overall, at the screening conducted in January 2003, 14 patients were positive for HBsAg, 105 patients were negative for HBsAg but positive for anti-HBs and/or anti-HBc, and 53 patients were positive for anti-HCV. There were no significant associations between positivity for anti-HEV IgG and the presence of serological markers of HBV or HCV (Table II).

TABLE I. Age-Specific Prevalence of Anti-Hepatitis E Virus (HEV) IgG Among 416 Patients on Maintenance Hemodialysis at Screening in January 2003, According to Gender and Optical Density (OD) Value of Anti-HEV IgG

Age (years)	No. of patients with anti-HEV IgG[a]			Anti-HEV IgG with high OD_{450} value of	
	Total	Male	Female	≥1.000	≥3.000
23–39	1/33 (3.0%)	0/21	1/12 (8.3%)	0/33	0/33
40–49	3/40 (7.5%)	3/29 (10.3%)	0/11	2/40 (5.0%)	1/40 (2.5%)
50–59	10/109 (9.2%)	9/71 (12.7%)	1/38 (2.6%)	4/109 (3.7%)	1/109 (0.9%)
60–69	17/138 (12.3%)	13/92 (14.1%)	4/46 (8.7%)	2/138 (1.4%)	0/138
70–79	6/77 (7.8%)	5/48 (10.4%)	1/29 (3.4%)	1/77 (1.3%)	0/77
80–91	2/19 (10.5%)	1/13 (7.7%)	1/6 (16.7%)	0/19	0/19
Total	39/416 (9.4%)	31/274 (11.3%)	8/142 (5.6%)	9/416 (2.2%)	2/416 (0.5%)

[a]Cut-off value for anti-HEV IgG was 0.152.

TABLE II. Characteristics of the Anti-HEV IgG-Positive and -Negative Patients at Screening Conducted in January 2003

Features	Anti-HEV IgG-positive (n = 39)	Anti-HEV IgG-negative (n = 377)	*P* value
Age (years)	62.4 ± 10.0	59.9 ± 12.8	0.1540 (NS[a])[b]
Duration of hemodialysis (years)	7.5 ± 7.0	7.6 ± 6.2	0.9671 (NS)[b]
Hepatitis B surface antigen (HBsAg)-positive	0	14 (3.7%)	0.4485 (NS)[c]
Anti-HBs/HBc-positive	8 (20.5%)	97 (25.7%)	0.6028 (NS)[c]
Anti-hepatitis C virus (HCV)-positive	4 (10.3%)	49 (13.0%)	0.8131 (NS)[c]

[a]NS, not significant.
[b]Welch's *t*-test.
[c]χ^2-test.

Prevalence of Anti-HEV IgG Among Hemodialysis Patients at the Start of Hemodialysis

The prevalence of anti-HEV IgG at the start of hemodialysis was surveyed retrospectively by testing stored serum samples that had been obtained from the 416 patients. Anti-HEV IgG was detected in 42 patients (10.1%), including 32 males and 10 females (Table III). Although two patients had anti-HEV IgG of extremely high OD_{450} value ($\geq$3.000), they were negative for both anti-HEV IgM and HEV RNA. Among the 42 patients with anti-HEV IgG at the first examination between January 1977 and October 2002, 35 patients remained seropositive and 7 patients tested negative for anti-HEV IgG in January 2003 (Table IV). On the other hand, among the 374 patients who were negative for anti-HEV IgG at the start of hemodialysis, 370 patients remained negative for anti-HEV IgG but 4 patients were found to be seropositive in the screening test performed in January 2003. In January 2003, 194 patients (46.6%) had a history of blood transfusion; however, the presence of a history of blood transfusion was not significantly associated with seropositivity for anti-HEV IgG.

Detection of HEV RNA in Four Patients who Contracted HEV Infection After the Start of Hemodialysis

The demographic characteristics of the four patients who contracted HEV infection during the observation period of 8.1–24.3 years, presence of a history of blood transfusion, and OD_{450} values of anti-HEV IgG in their serum samples that had been obtained at the start of hemodialysis and at the screening in January 2003 are shown in Table V. In the serum samples obtained in January 2003, the four patients had anti-HEV IgG with OD_{450} value of 0.416–1.348 but were negative for both anti-HEV IgM and HEV RNA. The stored serum samples that had been obtained semimonthly between 1978 and 2001 and monthly between 2002 and 2003 from each of the four patients (Patients 1–4), were tested for anti-HEV IgG and HEV RNA in order to clarify when they contracted HEV infection. Patient 1 was first positive for anti-HEV IgG on November 26, 1979 with an OD_{450} value of 1.381 and remained positive until the end of the observation period (Table VI). The serum sample containing anti-HEV IgG that had been obtained on November 26, 1979 was negative for HEV RNA; however, the serum sample that had been obtained

TABLE III. Age-Specific Prevalence of Anti-HEV IgG Among 416 Patients on Maintenance Hemodialysis at the Start of Hemodialysis, According to Gender and OD Value of Anti-HEV IgG

Age (years)	No. of patients with anti-HEV IgG[a]			Anti-HEV IgG with high OD_{450} value of	
	Total	Male	Female	$\geq$1.000	$\geq$3.000
14–29	2/27 (7.4%)	1/19 (5.3%)	1/8 (12.5%)	1/27 (3.7%)	0/27
30–39	2/52 (3.8%)	2/35 (5.7%)	0/17	1/52 (1.9%)	1/52 (1.9%)
40–49	7/83 (8.4%)	6/47 (12.8%)	1/36 (2.8%)	2/83 (2.4%)	1/83 (1.2%)
50–59	17/127 (13.4%)	15/89 (16.9%)	2/38 (5.3%)	2/127 (1.6%)	0/127
60–69	10/83 (12.0%)	5/57 (8.8%)	5/26 (19.2%)	2/83 (2.4%)	0/83
70–87	4/44 (9.1%)	3/27 (11.1%)	1/17 (5.9%)	0/44	0/44
Total	42/416 (10.1%)	32/274 (11.7%)	10/142 (7.0%)	8/416 (1.9%)	2/416 (0.5%)

[a]Cut-off value for anti-HEV IgG was 0.152.

TABLE IV. Characteristics of 416 Hemodialysis Patients, Stratified by the Presence/Absence of Anti-HEV IgG at the Start of Hemodialysis and the Screening of HEV Infection in January 2003

Presence of anti-HEV IgG at the start of hemodialysis/ at screening (January 2003)	No. of patients	Male (%)	Age (mean ± SD, years)[a]	Duration of hemodialysis[b] (mean ± SD [range], years)	No. of patients with a history of blood transfusion		
					Before the start of hemodialysis	After the start of hemodialysis	Either[c]
Yes/Yes	35	82.9	56.6 ± 11.9	6.3 ± 5.9 [0.3–21.8]	8 (22.9%)	10 (28.6%)	16 (45.7%)
Yes/No	7	42.9	50.6 ± 10.9	13.0 ± 6.9 [3–23.2]	1 (14.3%)	3 (42.9%)	3 (42.9%)
No/Yes	4	50.0	40.0 ± 7.8	18.2 ± 6.4 [8.1–24.3]	2 (50.0%)	4 (100%)	4 (100%)
No/No	370	64.9	52.3 ± 14.3	7.5 ± 6.1 [0.3–26.0]	98 (26.5%)	125 (33.8%)	171 (46.2%)
Total	416	65.9	52.5 ± 14.1	7.6 ± 6.3 [0.3–26.0]	109 (26.2%)	142 (34.1%)	194 (46.6%)

[a]At the start of hemodialysis.
[b]Patients who had been on maintenance hemodialysis for more than 3 months as of January 2003, were enrolled in the present study.
[c]Patients with a history of blood transfusion before the start of hemodialysis and/or after the start of hemodialysis.

14 days earlier (November 12, 1979) had detectable HEV RNA. Similarly, Patient 2 was first positive for anti-HEV IgG on April 21, 1980, with an OD_{450} value of >3.000 and continued to be positive thereafter. Patient 2 had detectable HEV RNA in the serum sample that had been obtained 14 days earlier (April 7, 1980). Patient 3 was first positive for anti-HEV IgG on August 16, 1988, with an OD_{450} value of >3.000 and continued to be positive thereafter. This patient had detectable HEV RNA in two consecutive serum samples, i.e., the serum sample obtained on the day of emergence of anti-HEV IgG (August 16, 1988) and the serum sample that had been obtained 14 days earlier (August 2, 1988). Patient 4 was first positive for anti-HEV IgG on January 6, 2003 with an OD_{450} value of 1.348 and continued to be positive until the end of the observation period. However, HEV RNA was not detectable in any of the stored serum samples of Patient 4, unlike the other three patients, and this was probably due to the lack of a serum sample obtained 14 days before the emergence of anti-HEV IgG: only a serum sample obtained 1 month earlier was available.

Surprisingly, although transient viremia was recognizable in three of the four patients who contracted HEV infection, anti-HEV IgM was not detected in any of the stored serum samples of the four patients, using not only an "in-house" ELISA but also a commercially available ELISA kit supplied by Genelabs. In support of this observation, anti-HEV IgA which can be utilized as an additional confirmatory antibody for recent HEV infection [Chau et al., 1993; Tokita et al., 2003], was not detected throughout the observation period in two patients (Patients 1 and 2) and was only weakly positive within a short period of time or at a single time point in the remaining two patients (Patients 3 and 4, respectively).

Detection of HEV RNA From Pilot Serum Samples of Transfused Blood Units

Patients 2–4 contracted HEV infection 1.5–8.1 years after the start of hemodialysis and had no history of blood transfusion within 1 year before seroconversion to anti-HEV IgG. However, Patient 1 who became positive for HEV RNA in the serum on November 12, 1979, approximately 1 month after initiation of hemodialysis, had received 2 U of blood on October 22, 1979 (3 weeks before detection of HEV RNA in the circulation). Two pilot serum samples of transfused blood units had been stored and were subjected to PCR assay. Of remarkable interest, one of the two pilot samples had detectable HEV RNA, although they were negative for anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA.

Genetic Analysis of HEV Isolates Recovered From Three Viremic Patients and an HEV RNA-Positive Pilot Sample

The three HEV isolates recovered from the transiently viremic patients (Patients 1–3) were named

TABLE V. Past History of Blood Transfusion and Anti-HEV IgG in Four Hemodialysis Patients who Became Seropositive for Anti-HEV IgG After the Start of Hemodialysis

Patient	Sex	Blood transfusion		At the start of hemodialysis			Duration (years)	At screening (January 2003)		
		Before the start of hemodialysis	After the start of hemodialysis	Date of sampling	Age (years)	Anti-HEV IgG (OD_{450} value)		Date of sampling	Age (years)	Anti-HEV IgG (OD_{450} value)
1	Male	No	Yes (1979/10/22)	1979/10/9	31	0.075 (−)	23.2	2003/1/6	54	1.037 (+)
2	Female	Yes (1978/8/14)	Yes (1983/8/11)	1978/10/2	39	0.089 (−)	24.3	2003/1/7	63	0.416 (+)
3	Female	Yes (1985/11/5)	Yes (1986/3/13)	1985/11/25	45	0.068 (−)	17.1	2003/1/14	62	0.496 (+)
4	Male	No	Yes (1999/10/4)	1994/12/12	50	0.012 (−)	8.1	2003/1/6	58	1.348 (+)

TABLE VI. Laboratory Parameters, Anti-HEV Antibody Levels and HEV RNA in Periodic Serum Samples Obtained From Four Hemodialysis Patients With Transient HEV Infection

Patient	Date of sampling	ALT (IU/L)	AST (IU/L)	Anti-HEV (absorbance at 450 nm)			HEV RNA
				IgG-class	IgM-class	IgA-class	
1	1979/10/9[a]	18	14	0.075 (−)	0.029 (−)	0.036 (−)	−
	1979/10/29	12	3	0.107 (−)	0.035 (−)	0.042 (−)	−
	1979/11/12	9	7	0.138 (−)	0.032 (−)	0.039 (−)	+
	1979/11/26	9	8	**1.381** (+)	0.045 (−)	0.085 (−)	−
	1979/12/10	6	8	**2.319** (+)	0.052 (−)	0.081 (−)	−
	1979/12/24	4	2	**2.619** (+)	0.042 (−)	0.090 (−)	−
	1980/1/7	10	6	**2.733** (+)	0.050 (−)	0.099 (−)	−
	1980/1/21	12	3	**2.705** (+)	0.047 (−)	0.110 (−)	−
	1980/2/4	14	5	**2.447** (+)	0.037 (−)	0.073 (−)	−
	1980/2/18	12	7	**2.666** (+)	0.040 (−)	0.086 (−)	−
	1980/4/14	1	1	**2.103** (+)	0.044 (−)	0.095 (−)	−
	1980/10/28	12	8	**1.517** (+)	0.053 (−)	0.079 (−)	−
	1987/1/12	35	22	**0.884** (+)	0.024 (−)	0.052 (−)	−
	1991/1/2	21	26	**0.837** (+)	0.016 (−)	0.014 (−)	−
	1997/1/13	14	15	**0.635** (+)	0.023 (−)	0.035 (−)	−
	2003/1/6[b]	18	14	**1.037** (+)	0.021 (−)	0.027 (−)	−
	2003/11/10	14	9	**1.093** (+)	0.021 (−)	0.031 (−)	−
2	1978/10/2[a]	7	1	0.089 (−)	0.069 (−)	0.031 (−)	−
	1980/3/24	23	20	0.087 (−)	0.056 (−)	0.032 (−)	−
	1980/4/7	4	9	0.087 (−)	0.045 (−)	0.029 (−)	+
	1980/4/21	6	7	>**3.000** (+)	0.078 (−)	0.093 (−)	−
	1980/5/5	6	6	>**3.000** (+)	0.088 (−)	0.096 (−)	−
	1980/5/19	1	1	>**3.000** (+)	0.057 (−)	0.083 (−)	−
	1980/6/2	5	6	>**3.000** (+)	0.076 (−)	0.093 (−)	−
	1980/6/16	5	5	>**3.000** (+)	0.081 (−)	0.091 (−)	−
	1980/6/30	7	6	>**3.000** (+)	0.076 (−)	0.084 (−)	−
	1980/8/11	2	5	**2.819** (+)	0.071 (−)	0.078 (−)	−
	1980/9/22	8	7	**2.804** (+)	0.060 (−)	0.059 (−)	−
	1981/2/2	1	6	**2.210** (+)	0.070 (−)	0.055 (−)	−
	1992/1/7	21	27	**0.483** (+)	0.031 (−)	0.116 (−)	−
	2003/1/7[b]	7	13	**0.416** (+)	0.012 (−)	0.063 (−)	−
	2003/11/11	10	15	**0.454** (+)	0.016 (−)	0.065 (−)	−
3	1985/11/25[a]	49	45	0.068 (−)	0.024 (−)	0.030 (−)	−
	1988/7/5	13	15	0.062 (−)	0.022 (−)	0.032 (−)	−
	1988/7/19	11	11	0.061 (−)	0.023 (−)	0.038 (−)	−
	1988/8/2	11	12	0.058 (−)	0.018 (−)	0.030 (−)	+
	1988/8/16	15	12	>**3.000** (+)	0.034 (−)	**0.664** (+)	+
	1988/8/30	14	12	>**3.000** (+)	0.082 (−)	**0.829** (+)	−
	1988/9/13	11	12	>**3.000** (+)	0.085 (−)	**0.654** (+)	−
	1988/10/11	10	10	>**3.000** (+)	0.079 (−)	**0.450** (+)	−
	1988/10/25	20	17	>**3.000** (+)	0.072 (−)	**0.398** (+)	−
	1988/11/22	10	13	>**3.000** (+)	0.044 (−)	0.318 (−)	−
	1989/3/6	11	14	>**3.000** (+)	0.034 (−)	0.167 (−)	−
	1989/5/29	13	14	**2.776** (+)	0.034 (−)	0.141 (−)	−
	1995/1/10	14	16	**0.807** (+)	0.023 (−)	0.039 (−)	−
	2000/1/2	14	16	**0.517** (+)	0.008 (−)	0.024 (−)	−
	2003/1/14[b]	10	15	**0.496** (+)	0.009 (−)	0.030 (−)	−
	2003/11/4	12	18	**0.349** (+)	0.009 (−)	0.023 (−)	−
4	1994/12/12[a]	15	21	0.012 (−)	0.017 (−)	0.028 (−)	−
	2002/11/11	9	14	0.064 (−)	0.027 (−)	0.054 (−)	−
	2002/12/9	11	11	0.061 (−)	0.028 (−)	0.057 (−)	−
	2003/1/6[b]	10	12	**1.348** (+)	0.107 (−)	**0.355** (+)	−
	2003/2/3	8	12	**1.575** (+)	0.131 (−)	0.343 (−)	−
	2003/3/3	9	9	**1.412** (+)	0.099 (−)	0.284 (−)	−
	2003/4/14	9	13	**1.180** (+)	0.067 (−)	0.262 (−)	−
	2003/11/10	7	12	**0.815** (+)	0.042 (−)	0.208 (−)	−

[a]Date of the start of hemodialysis.
[b]Date of the initial screening in the current study.

HE-JHD1979, HE-JHD1980, and HE-JHD1988, respectively, and the HEV isolate recovered from the HEV RNA-positive pilot serum sample that had been transfused to Patient 1 was named HE-JHD1979d. 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. The HE-JHD1979 and HE-JHD1979d isolates were 100% identical, supporting transfusion-transmitted HEV infection in Patient 1. The three HEV

isolates recovered from transiently viremic patients were 92.0–95.6% similar to each other, and were closely related to the prototype Japanese isolate of genotype 3 (JRA1, see accession no. in Fig. 1) with nucleotide sequence identity of 91.2–94.7%, and were only 79.3–80.5, 74.9–76.5, and 78.8–79.4% similar to the B1 isolate of genotype 1, MEX-14 isolate of genotype 2, and T1 isolate of genotype 4, respectively, in the 412-nt ORF2 sequence. The phylogenetic tree constructed based on the common 301-nt sequence within ORF2 sequence confirmed that the HE-JHD1979, HE-JHD1979d, HE-JHD1980, and HE-JHD1988 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 (HE-JA5, HE-JA6, HE-JA9, HE-JA11, HE-JA21, HE-JA23, HE-JF2, HE-JO-1982, JJT-Kan, and JRA1) and swine (swJ570 and swJ681), supporting the indigenous nature of these HEV isolates.

DISCUSSION

HEV is associated frequently with fecal-contaminated drinking water or poor sanitation conditions in developing countries, and blood transfusion is not considered to be an important cause of HEV transmission as the virus does not produce a chronic carrier state. However, the theoretical possibility of HEV transmission via a parenteral route in developing countries has been suggested due to the high endemicity of HEV; the fact that the majority of HEV infections were subclinical; and documentation of viremia during the incubation period of the disease [Arankalle and Chobe, 1999]. In fact, 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 able potentially to cause transfusion-associated hepatitis E in areas of high endemicity [Arankalle and Chobe, 1999, 2000]. Such a possibility is also supported in industrialized countries where HEV infection is now considered to be low-endemic, based on the observation that positivity for anti-HEV antibody was more frequent among transfusion recipients than among the same number of non-transfused controls [Mannucci et al., 1994]. However, the epidemiology of HEV infection among hemodialysis patients who have a high rate of a history of blood transfusion and are at increased risk for infection with

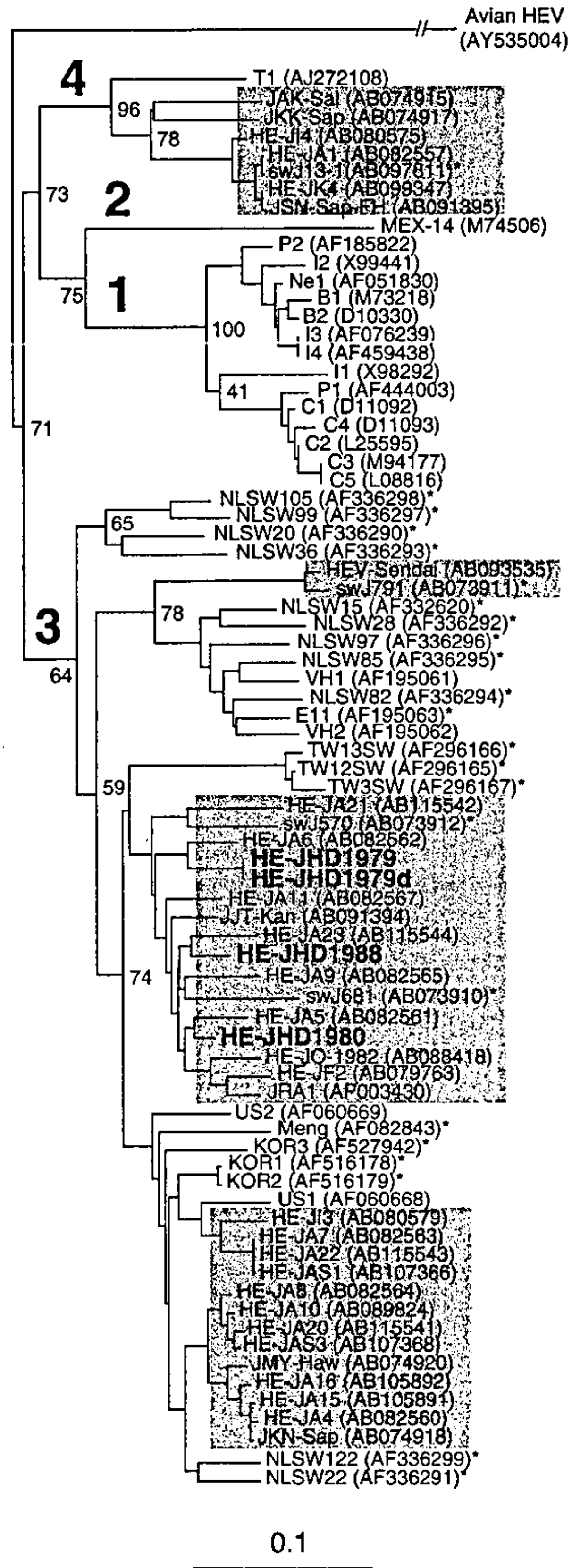

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the open reading frames (ORF)2 region (301 nt) of 77 hepatitis E virus (HEV) isolates, using an avian HEV (AY535004) as an outgroup. In addition to the HE-JHD1979, HE-JHD1979d, HE-JHD1980, and HE-JHD1988 isolates found in the present study which are indicated in bold type, 73 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 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 for visual clarity. Bootstrap values are indicated for the major nodes as a percentage obtained from 1,000 resamplings of the data.

blood-borne viruses, has not been fully understood and conflicting results have thus far been reported [Courtney et al., 1994; Halfon et al., 1994; Psichogiou et al., 1996; Fabrizi et al., 1997; Ding et al., 2003].

In the current study, at the first screening for HEV infection of hemodialysis patients at a dialysis unit of a city hospital in Japan conducted in January 2003, a high prevalence (9.4%) of anti-HEV IgG was observed among the 416 patients on maintenance hemodialysis who had been undergoing hemodialysis for 0.3–26 (mean, 7.6) years. However, we did not find significant associations between infection of HEV and infection with other blood-borne viruses such as HBV or HCV, consistent with a previous report [Fabrizi et al., 1997]. When the stored serum samples that had been collected at the start of hemodialysis from the 416 patients were tested for anti-HEV IgG, a high prevalence of 10.1% (42/416) was also observed, suggesting that the majority (89.7% or 35/39) of hemodialysis patients who were positive for anti-HEV IgG in January 2003, had been infected before initiation of hemodialysis. Furthermore, there was no appreciable difference in the prevalence of anti-HEV IgG at the start of hemodialysis between the patients who did or did not have a past history of blood transfusion (8.3% [9/109] vs. 10.7% [33/307], $P = 0.5776$), suggesting that the HEV infection in our hemodialysis patients that was acquired before the initiation of hemodialysis, had not been acquired by blood transfusion in the majority of cases. Of note, the prevalence of anti-HEV IgG at the start of hemodialysis tended to be higher among males than among females (11.7 vs. 7.0%), similar to the reported higher prevalence of anti-HEV IgG among males in the general population and the higher prevalence of HEV-associated hepatitis among male patients who had no history of blood transfusion within one or more years before the onset of disease [Tanaka et al., 2001; Mizuo et al., 2002]. Therefore, we would consider the possibility that our patients who were positive for anti-HEV IgG at the start of hemodialysis had acquired HEV infection in the community. It is likely that differences in the prevalence of HEV in the general population at the regional level, the criteria for inclusion of patients, and the routes of HEV transmission could partly explain the diverse results obtained in previous studies on hemodialysis patients. In Japan, regional differences in the prevalence of clinical and subclinical HEV infection have been reported [Li et al., 2000; Okamoto et al., 2003]. Furthermore, it was found previously that HEV-associated hepatitis was associated significantly with males, higher age (≥40 years) and living in the northern part of Japan [Mizuo et al., 2002]. It has been revealed that the zoonotic food-borne mode of transmission of HEV to humans may play an important role in the occurrence of hepatitis E [Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Tamada et al., 2004]. However, the mode of HEV transmission is unclear in 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]. Two male patients (49 and 52 years of age) were admitted in 2001 and 2002, respectively, to the hospital with our dialysis unit, and were diagnosed retrospectively as sporadic acute hepatitis E, but the mode(s) of HEV transmission could not be specified (unpublished observations). Further studies are needed to elucidate the region-dependent prevalence and mode(s) of clinical and subclinical HEV infection in the general population of Japan including Aichi Prefecture, where our dialysis unit is located.

In the present study, during the mean observation period of 7.7 years after the start of hemodialysis, four hemodialysis patients (1.1% or 4/374) acquired de novo HEV infection. In three of the four patients, the mode(s) of HEV transmission was unclear. However, it was found that the remaining one patient contracted HEV infection by transfusion of HEV-viremic blood in 1979. The HEV isolates, HE-JHD1979 and HE-JHD1979d, recovered from the stored serum sample from the patient and from the stored pilot serum of transfused viremic blood, respectively, were 100% identical in the 412-nt sequence of the ORF2 region, and segregated into genotype 3, and further into a cluster consisting of apparently Japan-indigenous strains. These results support our previous observation that a domestic HEV strain has been present for >2 decades in Japan [Aikawa et al., 2002] and also the recent report of a patient who was infected with HEV by transfused blood from a voluntary blood donor in Hokkaido, Japan [Matsubayashi et al., 2004], where clinical HEV infection is most prevalent. Although HEV infection via blood transfusion does not occur frequently as described above, we found a probable case of transfusion-transmitted HEV infection by molecular approaches. Therefore, the potential risk of post-transfusion hepatitis E should be taken into consideration even in low-endemic countries including Japan.

The four patients who contracted de novo HEV infection after the initiation of hemodialysis, did not have an elevated ALT level even after the appearance of anti-HEV IgG in the circulation, indicating that the HEV infection in the four patients was exclusively subclinical, although we cannot rule out the possibility of mild ALT elevation during the interval of 2 or 4 weeks. Of note, anti-HEV IgM was not detected in any of the four patients, and was also undetectable in all four patients by a commercially available kit: this may argue against the possibility of low sensitivity of the "in-house" ELISA system. It has been reported that IgA anti-HEV test can be utilized as an additional confirmatory test for recent HEV infection [Chau et al., 1993; Tokita et al., 2003]. IgA anti-HEV was also undetectable in two patients but was detectable weakly in the remaining two patients, suggesting that IgA anti-HEV detection is useful for serological diagnosis of acute HEV infection in the absence of IgM anti-HEV. As patients on maintenance hemodialysis have an impaired immune response [Goldblum and Reed, 1980; Girndt et al., 2001; Libetta et al., 2001], they may be unable to raise an adequate antibody response to viral proteins, especially in subclinical infection. Hemodialysis patients also have an

impaired immune response to HCV proteins or to hepatitis B vaccination [Rapicetta, 1992; Devesa et al., 1997]. Although the OD value of anti-HEV IgG in the four patients was lower than that among non-dialysis patients with clinical HEV infection, anti-HEV IgG persisted until the end of the observation period (as of June 2004, when this report was prepared) of 24 years in Patient 1, 24 years in Patient 2, 15 years in Patient 3, and 1 year in Patient 4. Therefore, we would like to consider that underestimation of HEV infection in our studied population by our anti-HEV IgG assay may be small or hopefully negligible.

In conclusion, it was found that approximately 90% of anti-HEV IgG-positive patients who were receiving maintenance hemodialysis for 0.3–26.0 (mean, 7.6) years had already been infected with HEV at the start of hemodialysis, regardless of the presence of a past history of blood transfusion before the initiation of hemodialysis, suggesting that the majority of anti-HEV IgG-positive hemodialysis patients had acquired HEV infection in the community through undefined route(s) but not via blood transfusion. However, a patient with probable transfusion-transmitted HEV infection was identified who was infected with an apparently Japan-indigenous HEV strain of genotype 3 21 days after transfusion of the implicated viremic blood in 1979. Based on the present study and the recent report by Matsubayashi et al. [2004], cases of transfusion-associated HEV infection may have been present for more than two decades in Japan. Therefore, the potential risk of post-transfusion hepatitis E should be taken into consideration even in HEV low-endemic countries including Japan.

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Transfusion-transmitted hepatitis E caused by apparently indigenous hepatitis E virus strain in Hokkaido, Japan

Keiji Matsubayashi, Yasuhiro Nagaoka, Hidekatsu Sakata, Shinichiro Sato, Kanji Fukai, Toshiaki Kato, Kazuaki Takahashi, Shunji Mishiro, Mitsunobu Imai, Naokazu Takeda, and Hisami Ikeda

BACKGROUND: In industrialized countries, sporadic cases of hepatitis E have been reported in individuals who have never been in an endemic area. Hepatitis E virus (HEV) infection commonly occurs via the fecal-oral route but a potential risk of transfusion transmission route has been suggested.
STUDY DESIGN AND METHODS: A 67-year-old Japanese male patient who had never been abroad received a transfusion of blood from 23 voluntary donors and developed acute hepatitis with unknown etiology after transfusion. His blood samples were tested for viral markers of hepatitis viruses.
RESULTS: HAV, HBV, HCV, CMV, and EBV were ruled out as causative agents in this case. The patient's blood sample in the acute phase contained HEV RNA as well as IgM and IgG anti-HEV. HEV RNA was also detected in one of the FFP units transfused. The donor had no history of traveling abroad and had a normal ALT level at the time of donation. The PCR products from the patient and the donor showed complete identity for two distinct regions of HEV within open reading frame 1.
CONCLUSION: The patient was infected with HEV via transfused blood from a volunteer donor. A potential risk of posttransfusion hepatitis E should be considered even in nonendemic countries.

Hepatitis E virus (HEV) is a major cause of epidemic hepatitis that is usually developed as acute hepatitis in endemic areas in Asia, Africa, Central and South America, and the Middle East.[1] Recent evidence indicates that, in industrialized countries, sporadic acute or fulminant hepatitis E occurs in individuals who have no history of traveling to HEV endemic areas[2-10] and that hepatitis E is a zoonotic disease; pigs and other animals appear to be linked to human infection as reservoirs.[11-18] In Japan, HEV infection has been rarely reported and has been considered as an imported infection from endemic areas for a long time. An epidemiologic study with a sensitive ELISA system, however, revealed that 2 to 14 percent of the healthy population in Japan was seropositive for the presence of IgG anti-HEV.[19] Approximately 13 percent of the non-A, -B, and -C acute hepatitis cases in Japan were caused by HEV.[9] Moreover, after the initial discovery and the characterization of indigenous Japanese strain, JRA1, from a patient with non-A, -B, and -C acute hepatitis, who had never been abroad,[7] several indigenous Japanese HEV strains were recovered from patients with acute or fulminant hepatitis of non-A, -B, and -C etiology.[8-10,20] Although the question of when the first HEV strain made inroad remains unsettled, it is likely that heterogeneous strains of HEV

ABBREVIATIONS: HEV = hepatitis E virus; nt = nucleotide(s).

From the Hokkaido Red Cross Blood Center, Sapporo; the Department of Internal Medicine, Nikko Memorial Hospital, Muroran; the Department of Medical Sciences, Toshiba General Hospital, Tokyo; the Department of Microbiology, Kanagawa Prefectural Public Health Laboratory, Chigasaki; and the Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan.

Address reprint requests to: Keiji Matsubayashi, MS, Hokkaido Red Cross Blood Center, 2-2 Yamanote, Nishi-ku, Sapporo, Japan 063-0002; e-mail: kmatsu@hokkaido.bc.jrc.or.jp.

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have already been circulating and HEV has recently been recognized as an important causative agent of sporadic hepatitis of non-A, non-B, non-C aetiology in Japan. In endemic countries where fecal-oral routes of HEV transmission are common, it is suggested that there is a potential risk of transfusion-transmitted hepatitis E, because HEV viremia is known to appear in the early stage of infection and a significantly higher seroprevalence was observed in transfused hemodialysis patients compared to blood donors.[21] In India, where HEV is endemic, two cases of transfusion-transmitted hepatitis E were reported but they were not confirmed by molecular approaches.[22] Here we report a probable case of transfusion-transmitted hepatitis E in Japan, where HEV had been believed not to be endemic. Sequence analysis showed the isolates of both donor and patient appeared to be identical.

MATERIALS AND METHODS

Characteristics of the patient

In July 2002, a 67-year-old Japanese male patient (S.K.), who had never been abroad, received a transfusion of blood products from 23 voluntary donors during open-heart surgery. Although he was discharged 24 days after the operation, he was hospitalized again for acute hepatitis of unknown etiology with elevated levels of ALT and AST and bilirubinemia. He was followed-up for 134 days after the operation and his blood samples were collected periodically and stored below −20°C until testing.

Transfused blood samples

Twenty-three blood products from the 23 voluntary donors, 14 FFP units, 8 RBC units, and 1 PLT unit, were transfused to the patient during the operation and their stored blood samples were examined virologically including for HEV RNA.

The Japanese Red Cross Blood Centers have implemented a storing system of blood samples for every unit of donated blood since September 1996 to assess adverse effects of transfusion. All of the samples are stored below −20°C until testing.[23]

Blood donor samples with elevated ALT levels

There were 559,545 blood donations in Hokkaido from October 2000 through April 2002. Of these, 15,285 (2.7%) were disqualified because of an elevated ALT of greater than 60 IU per L. Of these, 40 had an ALT level of greater than 500 IU per L and tested negative for the presence of HBV and HCV by NAT. Among them, the samples of 18 donors, 16 men and 2 women, were subjected to RT-PCR testing for the presence of HEV RNA. These samples were stored below −20°C until testing.

RT-PCR for HEV RNA detection

Detection of HEV RNA was performed by nested RT-PCR targeting two distinct regions within ORF1. For 365 nucleotides (nt) within the methyltransferase-coding region, corresponding to nt 105 to 469 of JRA1 strain,[7] RT-PCR was carried out as described previously by Takahashi et al.[8] and a template for direct sequencing was prepared by the second-round PCR with the sense degenerate primer M13/HE5-2 (5′-GTTTTCCCAGTCACGACGCCYT KGCGAATGCTGTGG-3′) and a mixture of antisense degenerate primers M13/HE5-3 (5′-CAGGAAACAGCTAT GACTCRAARCAGTARGTGCGGTC-3′) and HE5-6 (5′-CAGGAAACAGCTATGACTYAAAACAGTAGGTTCGATC-3′). M13 sequences for direct sequencing are underlined.

To amplify sequences within the hypervariable and proline-rich hinge region, corresponding to nt 2127 to 2464 of JRA1 isolate, seminested RT-PCR was performed as described above with the sense primer HE-V1 (5′-ACCTGGGAGTCAGCCAAT-3′) and the antisense primer HE-V2 (5′-AACCAAGTACACTCAGACTCAAAG-3′) for the first-round PCR and internal sense primer HE-V3 (5′-TATACTCGCACCTGGTCGG-3′) and HE-V2 for the second-round PCR.

Sequence analyses of PCR products

The amplification products were sequenced on both strands with a cycle sequencing kit (PRISM BigDye Terminator, Version 2, Applied Biosystems Japan Ltd, Tokyo, Japan) and a genetic analyzer (Prism Model 3100 or 3700, Applied Biosystems Japan Ltd). The PCR product of a 326-nt region was sequenced with M13 primers, M13/RV (5′-CAGGAAACAGCTATGAC-3′) and M13/M4 (5′-GTTTTCCCAGTCACGAC-3′). For the PCR product of the hypervariable region, the same primers for the second-round PCR were used for sequencing. The sequences determined were analyzed with computer software (GENETYX-Win, Version 5.2, Software Development, Tokyo, Japan). The sequences were aligned together with reported HEV strains with a computer program (CLUSTAL W, Version 1.8).[24] A phylogenetic tree based on the 326-nt region within ORF1 was constructed by the neighbor-joining method[25] and the final tree was obtained by a computer program (TreeView, Version 1.6.6).[26] Bootstrap values were determined by resampling 1000 of the data sets.

The nucleotide sequence data reported in this article will appear in DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB113303 and AB113311 for HRC-SK, AB113304 and AB113312 for HRC-IM, AB113305 for HRC-HE1, AB113306 for HRC-HE2, AB113307 for HRC-HE3, AB113308 for HRC-HE4, AB113309 for HRC-HE5, and AB113310 for HRC-HE6.

ELISA for HEV antibodies

Samples were tested for IgM and IgG antibodies to HEV by ELISA that used virus-like particles as antigen that were produced in baculovirus-infected insect cells.[19]

Assays for viral markers other than HEV

Antibody assays to viruses other than HEV were performed with commercially available kits: anti-HAV IgM (AxSYM HA-M, Version 2.0, Abbott Laboratories, North Chicago, IL), anti-CMV IgM (Celltite SEIKEN Cytomegalo, Denka Seiken Co. Ltd, Tokyo, Japan), anti-EBV IgM and IgG (Diagnostics VCA-Test BML IgG and IgM test, BML, Inc., Tokyo, Japan), anti-HCV (AxSYM HCV, Abbott Laboratories), anti-HBc (AxSYM HBcAb, Abbott Laboratories), and anti-HBs (AxSYM AUSAB, Abbott Laboratories). HBsAg was assayed with AxSYM HBsAg (Abbott Laboratories, North Chicago, IL). HBV DNA and HCV RNA were assayed with NAT probe assays (DNA Probe FR-HBV, REBIOGEN, Inc., Tokyo, Japan; and Amplicor GT HCV Monitor, Roche Diagnostics, Berkeley, CA, respectively), according to the instructions of each company.

RESULTS

Clinical course of the patient

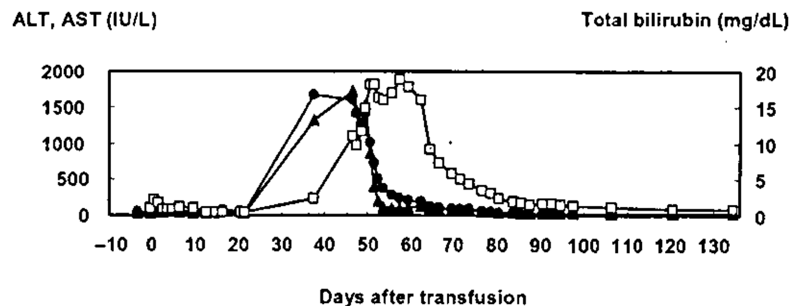
The clinical course of the patient (S.K.) is summarized in Fig. 1. When he was hospitalized again for acute hepatitis, he had an elevated ALT level of 1595 IU per L and an AST level of 1727 IU per L on Day 46 after transfusion; these normalized within 1 month, whereas the total bilirubin level rose to 11.0 mg per dL 2 weeks after the maximum ALT and AST elevation. His clinical state was improved 96 days after the transfusion. The retrospective testing of his blood sample from 4 days before the operation showed that he was negative for the presence of anti-HAV IgM, anti-CMV IgM, anti-EBV IgM, anti-HCV, HCV RNA, HBsAg, and HBV DNA.

In contrast, IgM and IgG class antibodies against HEV were detectable in his plasma sample on Day 37 after transfusion. HEV-RNA was detected from the serum sample of Day 37 and viremia lasted at least until Day 85 post-transfusion. HEV markers were not positive in his blood sample at 4 days before the operation. The IgG anti-HEV continued to be positive for 134 days after transfusion when last tested.

HEV testing of transfused blood

The 23 samples of transfused blood were tested for the presence of HEV RNA to determine whether the HEV was transfusion transmitted. HEV-RNA was detected in one of 23 samples of transfused blood to the patient. The donor I.M. a 24-year-old Japanese woman living in Hokkaido, had a normal ALT level of 10.0 IU per L at the time of donation and IgM- and IgG-class anti-HEV was not detectable (Table 1). FFP from this donor (I.M.) was transfused to the case patient (S.K.). The RBC product from the HEV-positive donation was transfused to another patient, Y.M. A following study revealed that patient Y.M., who had lymphoma showed no sign of hepatitis, clinically, virologically, or serologically of follow-up after transfusion. Neither HEV-RNA nor anti-HEV were detected in his blood 130 days after transfusion.

A blood sample from the HEV-positive donor's (I.M.) previous donation (15 months before the case donation) was available for testing. The sample was negative for the presence of HEV RNA or IgM- and IgG-class anti-HEV and had an ALT level of 8.0 IU per L. Five months after the case donation, the donor had seroconverted with IgM and IgG anti-HEV and HEV RNA was not detectable at that time (Table 1). On interview by telephone, donor I.M. had not been out of Japan during the incubation period and no



Day	-4	37	46	51	66	78	85	93	106	120	134
HEV RNA	–	+	+	+	+	+	+	–	NT	NT	NT
Anti-HEV IgM	–	+	+	+	+	+	+	+	+	+	+
Anti-HEV IgG	–	+	+	+	+	+	+	+	+	+	+

Fig. 1. Clinical course of the patient with hepatitis E and the testing results for HEV. The ALT (●), AST (▲), and total bilirubin levels (□) are also shown.

TABLE 1. Characteristics of the HEV-RNA-positive donor*

	ALT (IU/L)	HEV RNA	Anti-HEV IgM	Anti-HEV IgG
Previous donation (–15 months)	11	–	–	–
Case donation	10	+	–	–
Follow-up exam (+5 months)	8	–	+	+

* + = positive; – = negative.

clinical sign or symptoms of hepatitis during a follow-up of 5 months after the case donation.

HEV sequence study with PCR products from the donor and the patient

The PCR products of donor I.M. and patient S.K. were compared to each other for sequences corresponding to a 326-nt region encoding methyltransferase within the ORF1 of the HEV genome. The sequence (HRC-IM) of the PCR product from the transfused blood that was positive for the presence of HEV RNA showed complete identity with that (HRC-SK) from patient S.K.'s blood at 37 days after transfusion. According to Schlauder and Mushahwar's classification of HEV,[27] these isolates were segregated to genotype IV and were very similar to JKK-Sap and JSY-Sap,[8] which were isolated from hepatitis E patients living in Hokkaido (Fig. 2). JKK-Sap was different by only 1nt at position nt 261 and JSY-Sap by two nucleotides at the positions nt 261 and nt 330, based on the JKK-Sap sequence, respectively. The amino acid sequences were completely identical for these strains. Furthermore, for the 307-nt proline-rich hinge region of ORF1, the isolates from donor I.M. and patient S.K. showed complete identity.

Detection and analysis of HEV RNA in donors with elevated ALT

Six of the stored samples from 18 donors with elevated ALT levels higher than 500 IU per L, who were all men and aged 29 to 48 year, were positive for the presence of HEV RNA (Table 2). Phylogenetic analysis based on the 326-nt sequence of the ORF1 indicated that isolates of HRC-HE2, HRC-HE4, and HRC-HE5 were segregated to genotype III, and HRC-HE1, HRC-HE3, and HRC-HE6 as well as HRC-IM, to genotype IV (Fig. 2). All four strains of genotype IV were very closely related to each other with 99.4 to 100 percent identity in this region. HRC-HE1 and HRC-HE3 were completely identical with JKK-Sap. The addresses of HEV-positive donors were not concentrated in a particular area but widely distributed over Hokkaido.

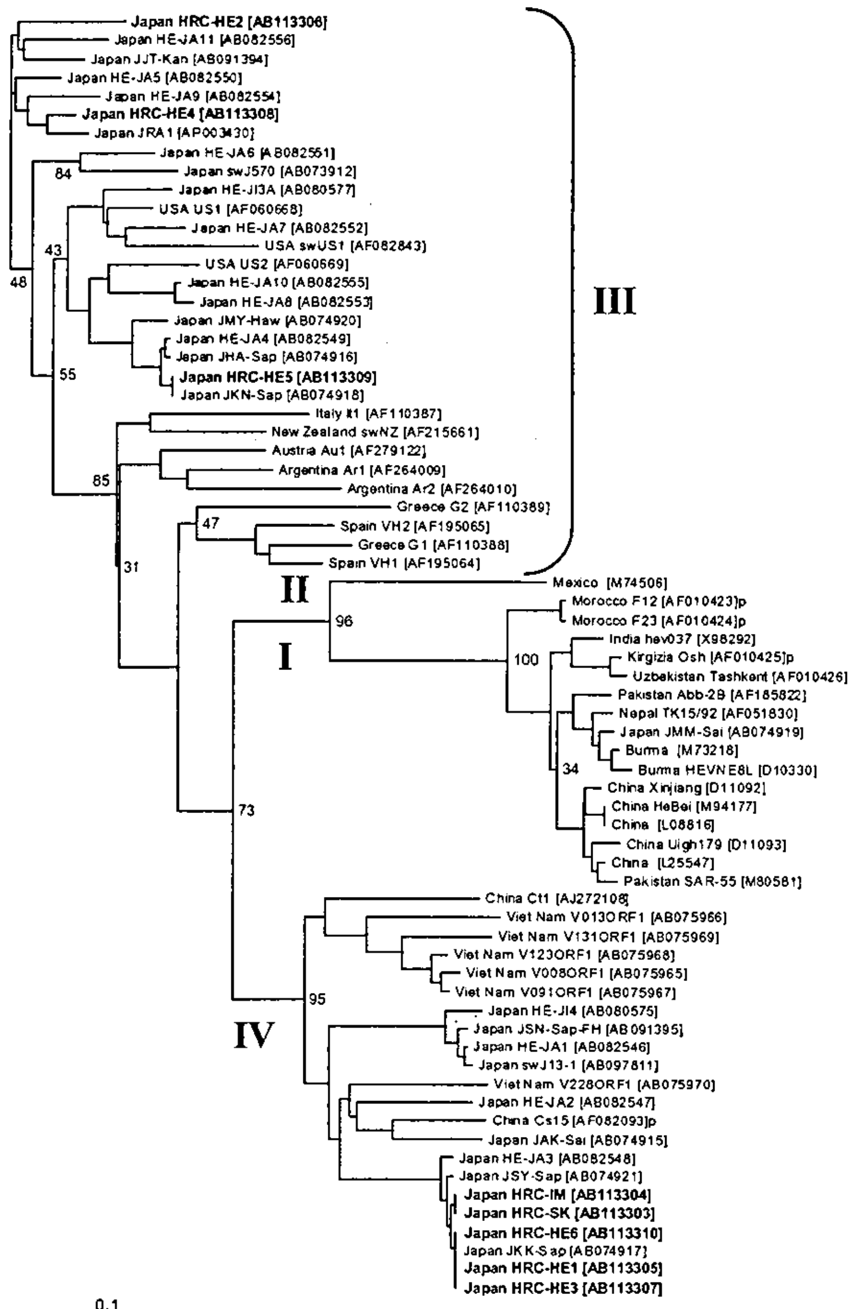


Fig. 2. Phylogenetic tree based on a 326-nt region of ORF1 for HEV strains derived from the case donor HRC-IM and patient HRC-SK and six donors with elevated ALT of greater than 500 IU per L. Accession numbers for the reference sequences are indicated parentheses. The isolated strains in this study are shown in boldface. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings.

Although HRC-HE1 and HRC-HE3 showed identical sequences, the two donors lived in different cities, and there was a time lag of 6 months between their donation dates.

Because some of the donors with a high ALT level were repeat donors, samples from different donations could be

TABLE 2. Characteristics of disqualified donors with elevated ALT† of greater 500 IU per L*

Donor	Date of donation	Age (years)	Sex	ALT level (IU/L)	Anti-HEV IgM	Anti-HEV IgG	HEV RNA	HEV strain (genotype)
1	October 2000	45	Male	878	−	−	−	
2	August 2000	29	Male	11	−	−	−	
	December 2000	29		767	+	+	+	HRC-HE1 (IV)
	August 2001	30		12	+	+	−	
	February 2002	31		19	+	+	−	
	May 2002	31		16	−	+	−	
3	December 2000	42	Male	558	−	−	−	
4	January 2001	32	Female	670	−	+	−	
5	March 2001	30	Male	506	+	+	+	HRC-HE2 (III)
6	April 2001	35	Male	1008	−	−	−	
7	April 2001	40	Male	1470	+	+	+	HRC-HE3 (IV)
8	June 2001	33	Female	545	−	−	−	
9	June 2001	36	Male	675	−	−	−	
10	July 2000	46	Male	21	−	−	−	
	July 2001	47		713	+	+	+	HRC-HE4 (III)
11	July 2001	31	Female	748	−	−	−	
12	August 2001	36	Male	1458	−	−	−	
13	August 2001	49	Male	647	−	−	−	
14	October 2001	39	Male	641	−	+	+	HRC-HE5 (III)
15	April 2000	47	Male	17	−	−	−	
	November 2001	48		740	+	+	+	HRC-HE6 (IV)
16	November 2001	40	Male	771	−	−	−	
17	December 2001	56	Male	531	−	−	−	
18	April 2002	33	Female	948	−	−	−	

* + = positive; − = negative.

studied. For all HEV-RNA-positive donors except for Donor 14, anti-HEV IgM was detected in the HEV-RNA-positive donations (Table 2). In the three HEV-infected donors, Donors 2, 10, and 15, ALT levels were normal and neither HEV RNA nor anti-HEV was detected in the previous donations. For Donor 2, HEV RNA was negative and ALT not elevated except for the HEV-RNA-positive donation; the HEV-positive donation had both IgG and IgM antibody to HEV and his anti-HEV IgM was still detectable 13 months after the HEV-positive donation.

DISCUSSION

We report the first case of transfusion-transmitted acute hepatitis E fully investigated by molecular approaches. The HEV-positive blood donor was asymptomatic and resident in Japan, where hepatitis E has been considered not endemic, and her donation was made in an early stage of HEV infection. Fecal-oral transmission is the common route of HEV infection in the outbreaks in endemic areas,[1] whereas little is known about the transmission routes for sporadic hepatitis E cases in industrialized countries. Vertical transmission as well as transfusion transmission has been suggested in endemic areas.[28]

In India, where hepatitis E is endemic, Arankalle and Chobe[22] reported two cases of transfusion-transmitted hepatitis E by means of retrospective analyses. Nevertheless, they were not successful in demonstrating the association of blood transfusion with hepatitis E infection by molecular approaches probably because of degradation of HEV RNA in the specimens during storage.

In our study, specimens from both the donors and the patient before and after the transfusion were available in good condition, which made it possible to determine that the blood transfusion was associated with HEV infection with molecular approaches.

The case patient was positive for both anti-HBc and HBsAg and negative for the presence of HBV DNA before and after transfusion, suggesting that he was not in an active HBV carrier state. Testing results for other five viruses regarding to hepatitis except for HEV showed that they were ruled out as a causative agent to this case. Based on the clinical data, he was diagnosed with acute hepatitis E. The case donor had a seroconversion of anti-HEV and appeared to be asymptomatic for HEV infection. The amplification products of two distinct regions of HEV corresponding to the methyltransferase gene and the hypervariable and proline-rich hinge domain of ORF1 from both the patient and the donor were sequenced, showing complete identity. Therefore, it is highly probable that the transfusion was responsible for the current hepatitis E case. In addition, the onset of the hepatitis was closely associated with the timing of the blood transfusion. The onset of the hepatitis was somewhere between 24 and 46 days after transfusion, which corresponded to the incubation period of 32 days in a case of transmission of HEV to a human volunteer.[29] Nevertheless, the possibility cannot be ruled out that the infection

occurred via other route and the sequence identity of the HEV in the donor and the patient was coincidental. RBCs, derived from the HEV-positive donation, did not appear to cause hepatitis E by transfusion to another patient. The patient had no clinical sign of hepatitis during follow-up after transfusion; neither HEV RNA nor anti-HEV were detected on the 130 days after transfusion but any other sample from the patient was not available for testing. The viral load of HEV in the RBC product could be too low to cause infection.

We also found six HEV-RNA-positive samples among donors with an ALT level of greater 500 IU per L in the same area, Hokkaido, and some of them had strains quite similar to the case strain, HRC-IM and HRC-SK.

Phylogenetic analyses of HEV isolates indicated a cluster of genotype IV indigenous to Hokkaido. The cluster includes highly homogeneous strains of genotype IV with 99.4 to 100 percent nucleotide sequence identities: JKK-Sap and JSY-Sap from hepatitis E patients; HRC-HE1, HRC-HE3, and HRC-HE6 from voluntary blood donors with an ALT level of greater than 500 IU per L; and HRC-IM from the case donor. Of three HEV strains of genotype III isolated in this study, HRC-HE5 showed very similar nucleotide sequence to JKN-Sap and JHA-Sap[8] isolated from acute hepatitis E patients. They all were derived from individuals living in Hokkaido. These results suggest that multiple HEV strains of genotype III and IV indigenous to Hokkaido may exist and are circulating there. It is interesting to note that a recently isolated swine HEV strain in Hokkaido showed 99 percent nucleotides homology over the entire genome with a human HEV strain of genotype IV.[16,17] Moreover, a direct evidence of HEV transmission from animal to human via uncooked deer meat was provided in Japan.[18] These support the idea that hepatitis E is a zoonotic disease and swine and deer are as reservoirs for human infection.

By implementation of sensitive HBV and HCV tests including NAT for donor screening, the residual risk of posttransfusion hepatitis B and C has become minimal.[30-32] Regarding other viruses associated with hepatitis such as HAV, HEV, CMV, and EBV, a specific test for each virus is not performed as routine donor screening in Japan. Although it may not be very effective in the early stage of infection or as a surrogate test for HBV or HCV infection,[33] ALT testing may be helpful in preventing posttransfusion hepatitis caused by other viruses associated with hepatitis. Approximately 8000 (2.3%) units of donated blood are disqualified yearly owing to an elevated ALT level of higher than 60 IU per L in Hokkaido. Forty donors showed ALT levels of higher than 500 IU per L in 1.5 years, of which at least six samples without HBV DNA nor HCV RNA were HEV-positive. It should be noted that the 6 donors were disqualified not by donor interviewing but by ALT testing. This suggests the possibility that asymptomatic HEV infection was present among other blood donors. Although the appropriate cutoff value might be reconsidered, ALT screening should not be discontinued because information about HEV infection is still poor and there is no other screening test to eliminate such asymptomatic HEV-positive donors in Japan.

In conclusion, although transfusion-transmitted HEV is probably much too rare to sustain HEV transmission in industrialized countries, where HEV infection is believed to be nonendemic, it should be taken into account that HEV is spread through uncertain routes, and the potential risk of transfusion-transmitted HEV infection should be considered. Further epidemiologic study is required to understand the current transmission routes of HEV infection.

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動物

豚のE型肝炎ウイルス

池 田 秀 利（動物衛生研究所）

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E型肝炎ウイルス（HEV, Hepatitis E virus）は日本の養豚だけでなく世界の養豚に広く感染していることが最近になって明らかになった。しかし、感染はしているものの、今のところいかなる「豚病」とも関係しているという証拠はない。現在問題になっているのは、豚のHEVがヒトに感染し、ヒトの急性E型肝炎の原因になっている可能性があることである。これを示唆する症例が、ごく少数例であるが、特に日本で見つかっていることに注目しなければならない。

HEVのヒトへの感染経路は主に糞口感染である。すなわち、ウイルスは汚染された水や食物を介して経口的にヒトに感染し、主に肝臓で増殖し、胆管経由で腸管に放出され、糞便に混じって排泄される。よく似た感染環をとるウイルスにA型肝炎ウイルスがあり、発症が急性肝炎に限定され慢性肝炎に移行しない性質なども似ている。ヒトのE型肝炎の多発地域は中国、インド、中近東、アフリカ、メキシコなど広く分布し、そこは一般的に衛生状態の良くない地域でもある（参考Webサイト1）。この地域では大規模な流行も散発的な発生もある。一方、非多発地域はヨーロッパ、北米（メキシコを除く）、アジアの一部（日本、韓国、台湾など）などで、低頻度の散発例があるのみである。つい数年前まで、日本で発症する急性E型肝炎患者は、これらの多発地を旅行中に感染し、帰国して発症した人達がほとんどであろうと考えられていた。しかし、HEVの研究が進み、ウイルス遺伝子や抗体の検出法が開発されると、そういった人達ばかりではなく、海外渡航歴のない人達の中にも急性E型肝炎患者がいることが分かってきた。そういった国内感染の場合、ウイルスの感染源が、動物、特に豚やイノシシ、の肉ではないかと疑われる事例が増えているというのが現状である。

1．E型肝炎ウイルスの種類とE型肝炎の多発地

HEVは比較的小型の球形粒子で、中に1本鎖約7300塩基のRNA遺伝子が含まれている。RNAの中には3つの蛋白質をコードする領域がある。以前はカリシウイルス（Caliciviridae）科に分類されていたが、現在はそれとは区別され、未分類となっている。HEVは培養細胞で増やすことが困難で、感染性ウイルスとしての一般的性質が十分調べられていない。HEVに対する抗体を持つ野生動物種が多数見つかっており（サル、ドブネズミ、ウシ、ネコ、イヌなど）、HEVないしHEVに近縁なウイルスが色々な動物種に感染していると想像されるが、現在までに検出されているウイルスは大まかに分けて2種類だけである。一つはヒト、ブタなどから分離されるHEVと、もう一つはニワトリから分離されたトリHEVである。トリHEVは米国では相当数のニワトリに感染していると見られ、しかもニワトリの肝炎脾腫症候群（hepatitis-splenomegaly syndrome）の病原体ではないかと言われているが詳細は不明である。トリHEVはサルへの感染性が認められず、ヒトへ感染する可能性は低いと考えられている。本稿では特に記載のない限り、HEVをヒト、ブタなどに由来するウイルスのことを指すこととする。

ヒト、ブタなどから分離されるHEVは、さらに4つの遺伝子型に分類されている（表1）。I型とII型は、前述の多発地域のE型肝炎ヒト患者から分離される。動物からは、I型がネパールのドブネズミから分離されたという一報告がある。III型は、E型肝炎非多発地域のうち北米、ヨーロッパ、日本、韓国のヒトやブタやイノシシから分離されている。IV型は、非多発地域の中でも東アジア（日本、台湾、中国、ベトナム）のヒトから、動物では東アジア（日本、台湾、中国、インド、インドネシア）のブタ、イノシシ、シカなどから分離されている。すなわち、III型、IV型に関しては、現段階の限られた情報から推察すると、ヒトでは非多発地に分布し、動物では全世界に分布しているようである。

図1は世界で分離されたHEV株の遺伝子系統樹で、ウイルス遺伝子間の近縁関係を表している。HEVが4つの遺伝子型に大きく枝分かれしている。この系統樹のIII型とIV型を見ると、ブタから分離されたHEVとヒトから分離されたHEVが区別つけがたいほど入り