A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis E virus via zoonotic food-borne route

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BACKGROUND: Five cases of transfusion transmission of hepatitis E virus (HEV) have been reported so far. The infection routes of the causative donors remain unclear, however. Also, the progress of virus markers in the entire course of HEV infection has not been well documented.

STUDY DESIGN AND METHODS: Nucleic acid testing was performed by real-time reverse transcription–polymerase chain reaction targeting the open reading frame 2 region of HEV. Full-length nucleotide sequences of HEV RNA were detected by direct sequencing.

RESULTS: Lookback study of a HEV-positive donor revealed that the platelets (PLTs) donated from him 2 weeks previously contained HEV RNA and were transfused to a patient. Thirteen relatives including the donor were ascertained to enjoy grilled pork meats together in a barbecue restaurant 23 days before the donation. Thereafter, his father died of fulminant hepatitis E and the other 6 members showed serum markers of HEV infection. In the recipient, HEV was detected in serum on Day 22 and reached the peak of 7.2 log copies per mL on Day 44 followed by the steep increase of alanine aminotransferase. Immunoglobulin G anti-HEV emerged on Day 67; subsequently, hepatitis was resolved. HEV RNA sequences from the donor and recipient were an identical, Japan-indigenous strain of genotype 4. HEV RNA was detectable up to Day 97 in serum, Day 85 in feces, and Day 71 in saliva.

CONCLUSION: A transfusion-transmitted hepatitis E case by blood from a donor infected via the zoonotic food-borne route and the progress of HEV markers in the entire course are demonstrated. Further studies are needed to clarify the epidemiology and the transfusion-related risks for HEV even in industrialized countries.

Hepatitis E virus (HEV) infection has been considered to occur mainly via fecal-oral transmission and is an important public health concern in developing countries.[1] In industrialized countries including Japan, cases have been rarely reported and hepatitis E has been regarded as an imported infectious disease from its endemic areas. Recently, however, increasing numbers of sporadic cases have been reported,[2-11] some of which resulted from infection via a zoonotic food-borne route by consumption of raw or undercooked meats of wild boar, wild deer, or farmed pig that was contaminated with HEV.[8-11]

In 2004, we reported the first molecularly confirmed case of transfusion transmission of HEV.[12] The infection route in the causative donor was not very clear, however. Thereafter, at least four cases of transfusion transmission of HEV have been reported in Japan, the United Kingdom,

ABBREVIATIONS: FAM = 6-carboxyfluorescein; HEV = hepatitis E virus; ORF = open reading frame; PSL = predonisolone; TAMRA = 6-carboxy-tetramethylrhodamine.

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and France,[13-16] where hepatitis E is nonendemic and HEV infection routes remained to be obscure.

Here, we report a case of acute hepatitis E caused by transfusion transmission from the donor who was infected with HEV via a zoonotic food-borne manner. To our knowledge, this is the first case in which the infection route of the causative donor has been confirmed. Also, in this report, we describe, for the first time, the virus kinetics and changes of anti-HEV in serum, prospectively monitored from latent period of infection until convalescence, accompanied by disease progression in the patient.

MATERIALS AND METHODS

Detection and quantitation of HEV RNA

For reverse transcription–polymerase chain reaction (RT-PCR) to detect HEV RNA in the samples, the following oligonucleotides were designed to detect 75 nucleotides of highly conserved sequence in the open reading frame (ORF) 2 region of all HEV genotypes: forward primer 5′-CGGCGGTGGTTTCTGG-3′, reverse primer 5′-AAGG GGTTGGTTGGATGAATA-3′, and mixed probes with a 5′-reporter dye (6-carboxyfluorescein, FAM) and a 3′-quencher dye (6-carboxy-tetramethylrhodamine, TAMRA) and FAM-5′-TGACAGGGTTGATTCTCAGCCCTTCG-3′-TAMRA, FAM-5′-TGACCGGGTTGATTCTCAGCCCTTC-3′-TAMRA, and FAM-5′-TGACCGGGCTGATTCTCAGCCC TT-3′-TAMRA (Sigma-Aldrich Japan, Tokyo, Japan). Nucleic acid was extracted from 200 μL of serum and saliva and from 100 μL of 10 percent (wt/vol) fecal suspension in saline with kits (QIAamp MinElute virus spin kit, Qiagen K.K., Tokyo, Japan; and SMITEST R&D-EX, Medical & Biological Laboratories, Nagoya, Japan). Before extraction, the samples were centrifuged at 6000 × *g* at 4°C for 10 minutes; thereafter the clear supernatant was subjected to nucleic acid extraction. Before RT-PCR, RNA preparation of feces was diluted at 10 times with nuclease-free water to reduce the effect of inhibitors. Twenty microliters of nucleic acid sample was used for each reaction. Each 50 μL of reaction mixture contained 25 μL of 2× RT-PCR kit master mix (QuantiTect Probe RT-PCR kit, Qiagen), 0.5 μL of RT mix (QuantiTect Probe RT-PCR kit, Qiagen), 400 nmol per L each of forward and reverse primer, and 67 nmol per L each of three probes. RT-PCR mixture was incubated at 50°C for 30 minutes and at 95°C for 15 minutes, followed by 50 cycles of 94°C for 15 seconds, and 60°C for 1 minute utilizing a thermocycler (Applied Biosystems 7500, real time PCR system, Applied Biosystems, Tokyo, Japan). HEV nucleic acid testing (NAT) was performed individually. The analytical sensitivity of the HEV NAT was determined to be 25 (13-166) copies per mL (with 95% confidence interval) by logistic analysis. HEV viral load was determined from standard curves generated by using 10^1 to 10^7 copies of HEV RNA per reaction. The HEV quantitation standard was generated by transcribing HEV cDNA of HEV ORF2 region that was cloned into a plasmid (pCRII-TOPO, Invitrogen, Carlsbad, CA), using the in vitro transcription kit (MAXIscript T7 high-yield transcription kit, Ambion, Austin, TX). Purified plasmid DNA was linearized with *Hin*dIII restriction endonuclease and transcribed to yield 717-nucleotide-long RNA transcripts containing 75-nucleotide target sequence.

Phylogenetic analysis of HEV isolates

Entire or nearly entire sequences of HEV isolates were determined as previously described by Takahashi and coworkers.[4] The sequences were aligned together with reported HEV strains with a computer program (CLUSTAL W, Version 1.8).[17] A phylogenetic tree based on the nearly entire HEV RNA sequence was constructed by the neighbor-joining method,[18] and the final tree was obtained by a computer program (TreeView, Version 1.6.6).[19] Bootstrap values were determined by resampling 1000 times of the data sets. The nucleotide sequences isolates HRC-HE14C, JST-KitAsa04C, and JTC-Kit-FH04L reported in this study have been assigned DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB291965, AB291966, and AB291959, respectively.

Detection of serum anti-HEV

Samples were tested for immunoglobulin M (IgM)- and immunoglobulin G (IgG)-class antibodies against HEV using a commercial enzyme-linked immunosorbent assay kit (Viragent HEV-Ab, Cosmic Corp., Tokyo, Japan).[5,20]

Alanine aminotransferase testing

Alanine aminotransferase (ALT) testing was carried out using transaminase-HRII Nisseki/GPT (Wako Pure Chemical Industries Ltd, Osaka, Japan) on an automatic analyzer (ACA5400, Olympus Corp., Tokyo, Japan).

RESULTS

A lookback study of a causative blood donor

Blood from a 39-year-old Japanese male on September 20, 2004, was disqualified because of the elevated ALT level at 236 IU per L and tested for hepatitis viruses because of the abnormal ALT result. His blood sample turned out to be positive for the presence of HEV RNA at 4.8 log copies per mL as well as anti-HEV IgM and IgG and negative for the presence of any marker of hepatitis B virus (HBV) or hepatitis C virus (HCV). A lookback study revealed that his donated blood on September 6, 2004, 2 weeks before the last donation, was positive for the presence of HEV RNA at 3.1 log copies per mL and negative for the presence of IgM- or IgG-class anti-HEV. The HEV isolate, HRC-HE14C, was classified as genotype 4 of a Japan-indigenous strain (Fig. 1). The blood (platelet [PLT] concentrate) donated on

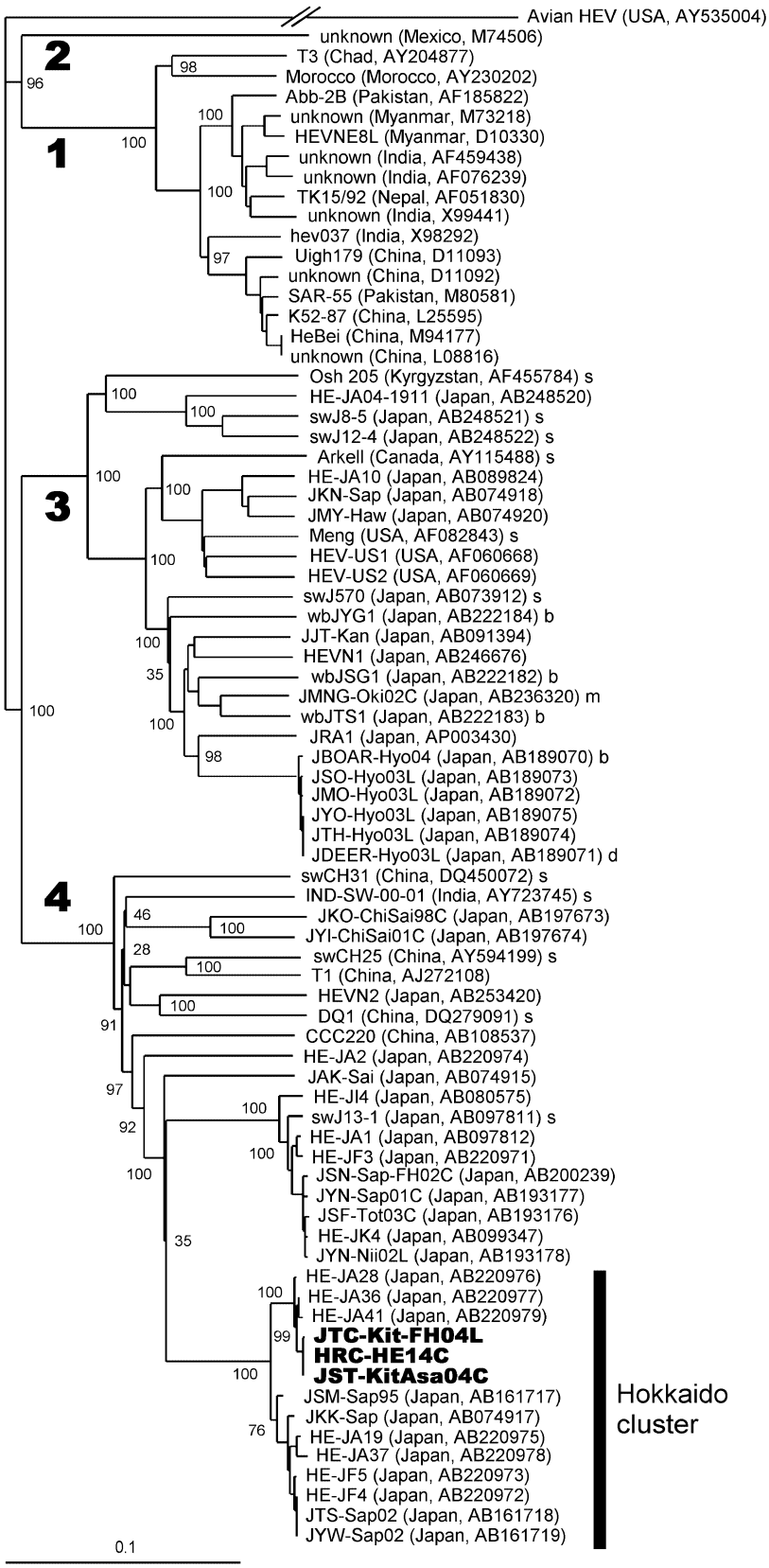

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the entire or nearly entire sequences of HEV genome of 77 isolates using an avian HEV (AY535004) as an outgroup. After the isolate name, the name of the country where the strain was isolated and accession numbers are shown in parentheses. The numbers 1, 2, 3, and 4 in bold indicated HEV genotypes. The 3 isolates HRC-HE14C from the causative donor, JST-KitAsa04C from the patient, and JTC-Kit-FH04L from the donor's father are indicated in bold. The letters "s," "b," "d," and "m" after parentheses denote HEV isolates from farmed pig, wild bore, wild deer, and mongoose, respectively. A vertical bar represents a cluster consisting of strains indigenous to Hokkaido, Japan. Bootstrap values are indicated for the major nodes as a percentage of the data set obtained from 1000 resamplings.

September 6 was released because it showed normal ALT and passed all the current blood screening tests. Transfusion was carried out 3 days after the blood donation, and the total amount of HEV in the PLT concentrate was estimated to be approximately 5.4 log copies. He was asymptomatic and did not feel tired or febrile in the periods near the two occasions of blood donation.

A minioutbreak of HEV infection in family members of the causative donor

Besides the causative donor, HEV RNA was detected in the blood of his 69-year-old father, who developed acute hepatitis on September 14, 2004, and finally died of fulminant hepatitis on October 14. Retrospective analysis of the father's blood sample taken on September 24, 41 days after the dining, revealed that the HEV strain, JTC-Kit-FH04L, was genotype 4. HEV RNA sequence analysis of the HEV isolates from the causative donor and his father showed only 9-nucleotide differences of 6588 nucleotides, suggesting that the two strains were extremely close but not identical (Fig. 1).

By retroactive interviewing, it was revealed that the causative donor and his 12 relatives gathered to enjoy grilled meats

including pig liver and intestines at a barbecue restaurant on August 14, 2004.[21] Blood samples from the relatives were tested for HEV markers with informed consent. Seven of the family members who ate grilled pig liver and/or intestines had IgM- and/or IgG-class anti-HEV in the blood samples taken 37 to 92 days after the barbecue party. Retrospectively, in the previous 6 months or more, dining out at that restaurant was the only occasion all the 13 relatives had eaten together.

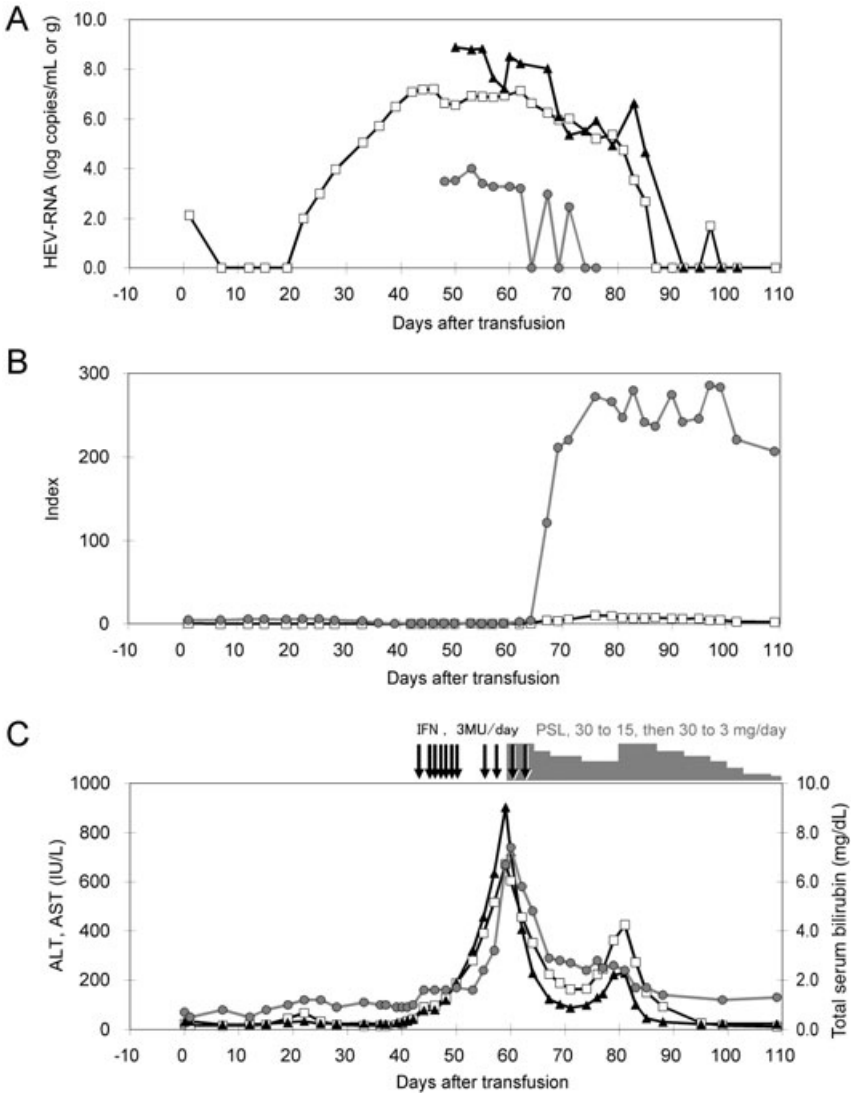

Fig. 2. Clinical course of transfusion-transmitted hepatitis E with kinetics of (A) HEV RNA, (B) serologic, and (C) biochemical markers after transfusion. The patient had transfusion of PLT concentrates contaminated with HEV on Day 0. (A) HEV RNA load was represented as log copies per mL of serum (□) or saliva (●) or per g of feces (▲). There were no data between Day 0 and Day 44 in feces and saliva. (B) Cutoff values of anti-HEV IgM (□) and IgG (●) antibodies are 30 and 13, respectively. (C) Medications were administered with IFN-α from Day 43 through Day 62 and with PSL from Day 59 through Day 112. (□) ALT; (▲) AST; (●) total serum bilirubin.

Clinical course of the patient

It was confirmed that the PLT concentrate (approx. 200 mL) contaminated with HEV was transfused to a 64-year-old Japanese male patient with non-Hodgkin's lymphoma on September 9, 2004, as shown Day 0 in Fig. 2. The patient had been treated with autologous peripheral blood stem cell transplantation accompanied with heavy chemotherapy since July 30, 2004. In the first 3 weeks after the transfusion, liver function tests sustained to be normal. On Day 22, the ALT level increased transiently at 67 IU per L, and HEV was detected in serum. While the ALT level returned to normal, the viral load in serum showed an exponential increase. Levels of aspartase aminotransferase (AST) and ALT took an upward turn on Day 41. There was no evidence for acute infection of hepatitis A virus, HBV, HCV, cytomegalovirus, or Epstein-Barr virus. He was diagnosed as acute hepatitis E. On Day 45, he was referred to the liver unit of Teine Keijinkai Hospital to treat presumed developing acute hepatitis E. Despite antiviral therapy with interferon (IFN) from Day 45, 2′,5′-oligoisoadenylate synthetase in serum never showed apparent increase and no obvious decrement of viral load had obtained (Fig. 2A). Levels of AST and ALT indicated creeping increase to reach highest levels of 903 and 673 IU per L on Day 59, respectively (Fig. 2C). The treatment was switched from IFN to predonisolone (PSL) in expectation of its anti-inflammatory effect. From Day 59 after induction of PSL treatment, AST and ALT showed rapid decrease and improvement of prothrombin time was observed (data not shown). Dosage of PSL was tapered gradually and discontinued on Day 113. Soon after anti-HEV IgG emerged on Day 67, HEV load in the serum sample had declined rapidly, although anti-HEV IgM in the serum sample remained negative (Figs. 2A and 2B). The levels in aminotransferases were normalized after Day 95 (Fig. 2C). The HEV strain JST-KitAsa04C detected in the patient was genotype 4 and its entire sequence analysis showed only a 1-nucleotide difference of 7255 nucleotides, suggesting the two isolates were identical (Fig. 1).

Serial quantitative changes of HEV load in serum, saliva, and feces of the patient

HEV RNA and anti-HEV were measured for every serum sample before and after the transfusion. In addition, HEV loads were also assessed prospectively for feces and saliva after his transference to the liver unit on Day 45. Any marker for HEV was not detected in serum sampled 37 days before the transfusion. A small amount of HEV RNA was transiently detected in his serum on Day 1, the next day of the transfusion. After the reappearance on Day 22, HEV RNA showed exponential increment with doubling every 29 hours and reached the peak level of 7.2 log copies per mL on Day 44. Beyond its plateau phase lasting 3 weeks, viral load revealed gradual decline over 2 weeks and thereafter decreased promptly. HEV viremia had been finally sustained for 63 days. HEV RNA remained detectable up to Day 97 in serum, Day 71 in saliva, and Day 85 in feces. Peak levels of HEV RNA were found on Day 53 in saliva at 4.0 log copies per mL and on Day 50 in feces at 8.9 log copies per g, respectively. HEV RNA was no longer detectable after Day 99 (Fig. 2A).

DISCUSSION

In Japan, a nonendemic country for hepatitis E, HEV infection is occurring more frequently than previously recognized. The prevalence of anti-HEV IgG in healthy Japanese persons ranged from 1.9 to 14.1 percent, depending on the geographic area,[20] and the prevalence of HEV RNA among Japanese blood donors with ALT level of at least 201 IU per L was 2.8 percent.[21] The risks of transfusion transmission of HEV might be low; however, five molecularly confirmed cases of transfusion-transmitted HEV infection have been reported in nonendemic countries so far.[12-16] In none of them, HEV infection routes of the causative donors are known. In this report, we have described the first case that the infection route of donor is clarified as zoonotic food-borne. The conclusion is based mainly on two observations.

First, by the epidemiologic study, the donor was determined to be infected in a minioutbreak of HEV infection in the context of food-borne transmission. Six of the 13 relatives who dined out together were positive for the presence of HEV RNA and/or IgM anti-HEV in their serum samples obtained 37 to 92 days after dining at the restaurant (Appendix 1). As for 4 relatives who were positive for the presence of IgM anti-HEV, HEV viremia might have transiently occurred without any symptom and had subsided by the time when blood samples were taken. Since IgM anti-HEV are regarded as the markers of acute HEV infection besides HEV RNA,[10] these facts strongly suggest that family members had recently become infected with HEV probably at the same time and remained asymptomatic. The party at the barbecue restaurant was the only opportunity all the 13 members had eaten together in the estimated period of HEV infection, 2 to 10 weeks.[22,23] Although it was difficult to identify the source of infection because no meat was left, they ingested various kinds of pig meats including liver and intestines, according to the replies to the questionnaire from the family members.[24] From this retrospective research, it is strongly suspected that the family members shared the motive of infection with HEV by ingestion of pig liver and intestines. In Japan, HEV has been isolated from farmed pigs,[9,25] wild deer,[8,26,27] and wild boar[10,11,26,27] as well as humans and recent studies also indicated that HEV is moderately resistant to heat inactivation.[28,29] Some reports suggest that a number of hepatitis E cases in Japan may be via a zoonotic food-borne route.[8-11,25-27,30]

Second, a single transmission route of HEV in this minioutbreak is corroborated by molecularly confirmed facts. From full-length sequence analysis, HEV RNAs detected in the donor and recipient were identical and closely related to that in his father. Among the strains of genotype 4 indigenous to Hokkaido, Japan, these three strains were segregated into a distinct cluster with a bootstrap value of 99 percent in a phylogenetic tree based on the entire or nearly entire sequences of HEV genome. Moreover, when comparing 412-nucleotide sequences (nucleotides 5985-6396 of HRC-HE14C) of ORF2 region, where many sequences of Japanese swine HEV are retrievable in DDBJ/EMBL/GenBank nucleotide sequence databases, high similarity (409/412 nucleotides, 99.3%) was observed between the HEV sequences derived from the causative donor and his father and strain swJL145 (AB105902),[9] which was detected in pig liver sold at a drug store in Hokkaido, Japan.

To date, in acute hepatitis E including transfusion transmission cases, dynamic relationships between infection markers for HEV and disease progression throughout the course from HEV transmission to convalescence of disease have not been demonstrated. This is the first case of acute hepatitis E, in which HEV kinetics in serum as well as in feces and saliva were described by using quantitative RT-PCR for HEV RNA from transfusion up to the end of viremia accompanied by disease progression, and the emergence and increase of anti-HEVs. In the current case, HEV viremia had lasted for 9 weeks or more and viral load reached its peak 15 days before the peak of aminotransferase level and died out promptly right after the appearance of anti-HEV IgG on Day 67. The results led us to understand the chronologic relationship between preceding viremia and after emergence and increase of anti-HEV.

Besides serum, the kinetics of HEV load in feces and saliva were concomitantly observed for the first time in hepatitis E in humans. After the transmission, HEV RNA remained detectable until Day 71 in saliva and Day 85 in feces. Among sera, saliva, and feces, every time point at peak viral loads resembled each other, 50 to 60 days after transmission. These facts may indicate that viral loads in

saliva and feces would also reflect viremia state. In addition, the results for saliva suggest that besides fecal-oral route, oral-oral transmission manner can be another route of human-to-human infection of HEV.

Soon after the transference to liver unit in the hospital, IFN-α therapy was started against HEV infection, indicating the exponential increase of viral load in sera. The levels in 2′,5′-oligoadenylate synthetase, however, induced by IFN and regarded as a predictive marker for favorable IFN efficacy,[31] did not show sufficient increase in serum (data not shown), and HEV load monitored concomitantly indicated no actual decrement during treatment. Thereafter, single-nucleotide polymorphisms in markers predicting the therapeutic efficacy of IFN, such as mannose-binding lectin,[32] MxA,[33] LMP7,[34] and osteopontin,[35] were examined, and all of them did not show the phenotype associated with favorable efficacy of IFN (data not shown).

Throughout his clinical course, no distinct positive result for IgM anti-HEV was observed. It is possible that the concentration of IgM anti-HEV was too low to be detected by the method we used. In fact, some of his samples showed equivocal reaction. Furthermore, underlying disease and the preceding treatment including autologous peripheral blood stem cell transplantation and large dosage chemotherapy might have led the patient to an immunocompromised state that responds inadequately for HEV infection. In fact, both serum levels in IgG and IgM had been indicated consistently less than lower limitation of normal ranges in the entire course (data not shown).

We should note that the present case was not revealed if the two practices had not been introduced, which are not widespread outside Japan. They are ALT screening and donor blood sample repository system. As a safety measure, the Japanese Red Cross Blood Center introduced ALT testing for a surrogate marker for non-A, non-B hepatitis virus infection. Because ALT testing contributes little for HCV infection after HCV antibody testing started, ALT screening has been discontinued in the United States and some other countries. Although the cutoff value may need to be reevaluated, the current case suggests that ALT testing may contribute to excluding blood with the presence of HEV. On the other hand, the Japanese Red Cross has established storing repository samples of all donations since 1996. Blood samples are collected from each donation and stored for 10 years at –30°C to investigate for lookback study such as the suspected cases of transfusion-transmitted infection and alloantibodies for TRALI. This system plays a very important role in the hemovigilance system in Japan.[36,37]

In the present case of transfusion-transmitted acute hepatitis E, the infection route in the blood donor was, for the first time, clarified to be zoonotic food-borne manner. In addition, the entire course including incubation period and disease progression in acute HEV infection was followed by serologic and virologic markers, and the patient was treated by monitoring them. To our knowledge, this is the first report for acute HEV infection in humans, in which various infection markers were prospectively monitored simultaneously with disease progression, excepting experimental hepatitis E in a volunteer.[38]

Our data suggest that hepatitis E is likely caused by consumption of contaminated pig meat, and there is a risk of transfusion transmission of HEV in Japan. The most effective preventive measure to reduce the risk of blood-borne transmission is to screen the blood supply for HEV or to implement pathogen inactivation. The epidemiology and the transfusion-related risks for HEV infection have not been fully understood in industrialized countries including Japan. We are undertaking epidemiologic studies of HEV infection in Japanese blood donors and a feasibility study of NAT screening for HEV in Hokkaido, Japan.

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APPENDIX 1

HEV infection markers in the 13 family members who participated in the dinner on August 14, 2004

Number*	Age (years)	Sex	Days after Aug 14, 2004	ALT (IU/L)	HEV markers		
					RNA (10^n/mL)	IgM† (index)	IgG‡ (index)
1	39	Male	23	27	+(3.1)	–(3.4)	–(2.0)
			37	236	+(4.8)	+(60.4)	+(14.2)
			49	70	+(2.1)	+(269.5)	+(154.7)
			53	44	–	+(257.8)	+(150.5)
			77	20	–	+(174.6)	+(163.0)
2	69	Male	41	1511	+(2.6)	+(187.2)	+(271.4)
3	43	Male	92	34	–	+(174.7)	+(297.7)
4	68	Male	79	15	–	+(51.7)	+(283.3)
5	37	Female	79	13	–	+(110.9)	+(90.3)
6	15	Male	90	17	–	+(63.3)	+(250.6)
7	58	Female	79	25	–	–(4.0)	+(25.9)
8	67	Female	79	15	–	–(1.4)	–(12.9)
9	38	Female	89	12	–	–(6.1)	–(1.1)
10	15	Male	77	19	–	–(0.3)	–(0.5)
11	14	Male	77	19	–	–(7.5)	–(0.3)
12	46	Male	90	15	–	–(2.2)	–(0.4)
13	6	Female	90	15	–	–(26.6)	–(1.1)

Data shown were originally reported by Kato et al.[24] without describing quantitative test results of antibodies and viral RNA and follow-up data of the causative donor.
* Number 1 is the causative donor; Number 2 is the donor's father and died of hepatitis E; others are their relatives.
† Positive ≥30 index.
‡ Positive ≥13 index.