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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.[34] 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.

A nationwide survey for hepatitis E virus prevalence in Japanese blood donors with elevated alanine aminotransferase

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BACKGROUND: Although we reported two cases of transfusion-transmitted hepatitis E in Japan, the prevalence of hepatitis E virus (HEV) in Japanese blood donors is not very clear.

STUDY DESIGN AND METHODS: Blood samples of donors who were deferred from donation because of elevated alanine aminotransferase (ALT) levels were collected from all Japanese Red Cross Blood Centers and subjected to HEV tests.

RESULTS: Among the 41 donors with elevated ALT levels higher than 500 IU per L in Hokkaido, HEV RNA was detected in 8 (19.5%) samples. In 1389 donor samples with ALT levels of higher than 200 IU per L in nationwide Japan, the numbers of positive HEV RNA, immunoglobulin M (IgM) anti-HEV, and immunoglobulin G (IgG) anti-HEV samples were 15 (1.1%), 14 (1.0%), and 45 (3.2%), respectively. Although RNA-positive donors were predominantly male and found in any geographic area of Japan, they tended to be higher in number in eastern Japan including Hokkaido and lower in number in western Japan. Of the 23 HEV-positive samples, 19 were Genotype 3 and 4 were Genotype 4. DNA sequences of the 9 isolates showed more than 98.5 percent homology with the known swine HEV isolates. In 1062 donor samples with ALT levels of 61 to 199 IU per L, the percentages of IgM and IgG anti-HEV–positive samples were 0.1 and 2.7 percent, respectively, although there was no HEV RNA–positive sample.

CONCLUSION: HEV markers (HEV RNA and anti-HEV) were detected in donors with elevated ALT levels who were widely distributed over Japan. The prevalence and incidence were higher in eastern Japan than in western Japan.

Although hepatitis E virus (HEV) is an emerging pathogen of enterically transmitted viral hepatitis in endemic areas, its infection is now recognized as a form of zoonosis in which swine, wild boar, and deer act as reservoirs for human infection in Japan.[1-8] HEV subgenomic sequencing studies have revealed a close relationship between the strains infecting humans and those infecting pigs. Accumulating evidence suggests that eating undercooked meat and viscera of pig and other animals is associated with a high risk of acquiring HEV infection. The HEV-infected individuals show transient viremia, which suggests the potential risk of a blood-borne route of HEV infection.[9-12] We previously reported two cases of transfusion-transmitted acute hepatitis E in Hokkaido, Japan.[9,12] In both cases, sequence analyses showed that the isolates of both donors and patients appeared to be identical. Moreover, HEV RNA has been reported to be present among some blood donors with elevated alanine aminotransferase (ALT) levels in Japan.[9,13,14] Although HEV was previously considered to be endemic only in developing countries, approximately 13 percent of the non-A, non-B, and non-C acute hepatitis cases were caused by HEV in Japan, a developed country.[15] However, no report has been available on a nationwide survey for HEV prevalence in Japan.

ABBREVIATIONS: B19 = human parvovirus B19; EBV = Epstein-Barr virus; HAV = hepatitis A virus; HEV = hepatitis E virus; JRC = Japanese Red Cross; RT = room temperature.

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Here we report the results of two studies. First, we studied the presence of HEV in plasma samples collected from blood donors showing extremely high ALT levels in Hokkaido, Japan. Subsequently, we expanded the area of investigation to nationwide and studied HEV prevalence in Japanese blood donor samples with elevated ALT levels obtained from all Japan.

MATERIALS AND METHODS

Blood donor samples with elevated ALT levels in Hokkaido

For the preliminary study, we studied the blood donors with elevated ALT levels of 500 IU per L and greater in Hokkaido. There were 1,049,566 blood donations in Hokkaido from April 2000 through March 2003. Of these, 23,827 (2.3%) were disqualified because of an elevated ALT level of 61 IU per L or greater, which was cutoff value in the Japanese Red Cross (JRC). Of these, 41 had an ALT level of 500 IU per L or greater (Table 1). The samples from these 41 donors enrolled in this study were stored below −20°C until testing. The tests for qualitative HEV RNA and/or for antibodies were performed as described below.

Blood donor samples with elevated ALT levels in nationwide Japan

All donor samples (n = 1389) with ALT levels higher than 200 (mean ± standard deviation [SD], 314 ± 249) IU per L were collected from all JRC Blood Centers over Japan between April 2003 and March 2004. In addition, 1062 donor samples with ALT levels of 61 to 199 IU per L were collected randomly from 3 blood centers (Hokkaido, Hiroshima and Fukuoka). The 47 blood centers were divided into eastern Japan (three blocks: Hokkaido, Miyagi, and Tokyo) and western Japan (four blocks: Aichi, Osaka, Okayama, and Fukuoka; Fig. 1). Hiroshima and Fukuoka blood centers belong to western Japan. The samples were subjected to real-time reverse transcription–polymerase chain reaction (RT-PCR) testing for the presence of HEV RNA and enzyme-linked immunosorbent assay (ELISA) for antibody tests against HEV as described below. The samples were kept frozen below −20°C until testing.

Real-time RT-PCR for HEV RNA detection and sequence analyses

Total nucleic acids were extracted from 200 μL of plasma sample using a virus spin kit (QIAamp MinElute, Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. The 20-μL eluate was subjected to one-step real-time RT-PCR and quantitative assay for HEV RNA as described in our previous study.[12] The amplification products were then sequenced directly on both strands and were analyzed as described previously.[16] The amplification products of ORF2 (412 nucleotides) from HEV RNA–positive samples were sequenced and compared with those of reported swine HEV isolates from pigs or pig livers by using GenBank Basic Local Alignment Search Tool (BLAST) homology search at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov).

The nucleotide sequence data reported in this article will appear in DDBJ/EMBL/GenBank nucleotide sequence databases with the Accession Numbers AB434132 for HRC-HE1, AB434133 for HRC-HE2, AB434134 for HRC-HE3, AB434135 for HRC-HE4, AB434136 for HRC-HE5, AB434137 for HRC-HE6, AB434138 for HRC-HE7, AB434139 for HRC-HE8, AB434140 for HRC-HE9, AB434141 for HRC-HE10, AB434142 for HRC-HE11, AB434143 for HRC-HE12, AB434144 for JRC-HE1, AB434145 for JRC-HE2, AB434146 for JRC-HE3, AB434147 for JRC-HE4, AB434148 for JRC-HE5, AB434149 for JRC-HE6, AB434150 for JRC-HE7, AB434151 for JRC-HE8, AB434152 for JRC-HE9, AB434153 for JRC-HE10, and AB434154 for JRC-HE11.

TABLE 1. ALT-disqualified donors from April 2000 through March 2003 in Hokkaido, Japan (total number of donors, 1,049,566)

	Number of donors with each ALT level (IU/L)						
Donors	61-99	100-199	200-299	300-399	400-499	500-	Total
Male	16,809	3,714	226	35	11	29	20,824
Percent*	88.1	85.8	78.7	60.3	52.4	70.7	87.4
Percent†	1.60	0.35	0.02	0.00	0.00	0.00	1.98
Female	2,281	616	61	23	10	12	3,003
Percent*	11.9	14.2	21.3	39.7	47.6	29.3	12.6
Percent†	0.22	0.06	0.01	0.00	0.00	0.00	0.29
Total	19,090	4,330	287	58	21	41	23,827
Percent†	1.82	0.41	0.03	0.01	0.00	0.00	2.27
Percent‡	80.1	18.2	1.2	0.2	0.1	0.2	100.0

* Rate relative to the donors with each ALT level, showing the ratio of sex difference.
† Rate relative to the total donors (1,049,566).
‡ Rate relative to the ALT-disqualified donors (23,827).

ELISA for HEV antibodies

Purified HEV Genotype 1 virus-like particles derived from recombinant baculovirus-infected insect cells were used as antigens for detection of antibodies to HEV.[17,18] HEV RNA–positive samples from 41 donors enrolled in the preliminary study were assayed by commercial HEV antibody ELISA kit (Cosmic Corp., Ltd., Tokyo, Japan) which basically consisted of the recombinant ORF2 protein as the antigen according to the manufacturer's protocol. In the subsequent study of all samples (n = 1389 and 1062) from all areas of

Fig. 1. Map of Japan showing the locations of seven geographic blocks. The 47 blood centers were divided into eastern Japan (three blocks: Hokkaido, Miyagi [six prefectures], and Tokyo [nine prefectures]) and western Japan (four blocks: Aichi [eight prefectures], Osaka [six prefectures], Okayama [nine prefectures] including Hiroshima prefecture, and Fukuoka [eight prefectures] including Fukuoka prefecture).

Japan, ELISA was performed as follows. Wells of microplates (Number 2592, 96-well Stripwell, flat bottom, Corning Life Sciences, Corning, NY) were coated with 50 μL of the recombinant ORF2 protein (3 μg/mL in phosphate-buffered saline [PBS]), and the plates were incubated at room temperature (RT) for 2 hours followed by incubation with 100 μL of blocking buffer containing 40 percent (vol/vol) calf serum (Gibco-BRL, Tokyo, Japan) at RT for 1 hour. The blocking buffer was discarded, and each well was washed five times with 450 μL of washing buffer (0.05% Tween 20 in PBS). To test for anti-HEV immunoglobulin G (IgG), 50 μL of each sample was added to each well at a dilution of 1:100 in saline containing 40 percent calf serum. The microplates were incubated at RT for 1 hour and then washed five times with washing buffer. Fifty microliters of horseradish peroxidase–conjugated goat anti-human IgG (IGB22; Institute of Immunology Co., Ltd., Tokyo, Japan; 1:2000) or immunoglobulin M (IgM; IGM49, Institute of Immunology Co., Ltd.; 1:500) in PBS containing 25 percent (vol/vol) fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) was added to each well and incubated at RT for 1 hour. The wells were washed five times with washing buffer. Fifty microliters of tetramethylbenzidine soluble reagent (Dako Co., Ltd., Carpinteria, CA) as a substrate was added to each well. The plate was incubated at RT for 10 minutes in the dark, and then 50 μL of 1 N sulfuric acid (Kanto Chemical Co., Inc., Tokyo, Japan) as tetramethylbenzidine stop buffer was added to each well. The optical density (OD) of each sample was read at 450 nm. Test samples with OD values equal to or greater than the cutoff value were considered positive for the presence of anti-HEV IgG or anti-HEV IgM in this ELISA. ODs of 0.18 [mean (0.019) + 7 × SD (0.024)] for anti-HEV IgG, and that of 0.19 [mean (0.022) + 6 × SD (0.028)] for anti-HEV IgM were used as the cutoff values. Reactive samples were tested by another HEV antibody ELISA kit (Cosmic) described previously. Samples were determined as positive if they were reactive by both ELISA methods.

Statistical analysis

A two-sided Fisher's exact test was used to compare the percentages of subjects with each HEV marker in the two geographic groups (eastern Japan vs. western Japan) or two age groups (10s-30s vs. 40s-60s).

RESULTS

Prevalence of HEV RNA in donors with elevated ALT levels in Hokkaido

In the primary study, more than 98 percent of those disqualified donors had an ALT level of less than 200 IU per L and more than 87 percent were male (Table 1). The number of donors with elevated ALT levels higher than 500 IU per L was 41 (0.2%). Among the 41 donors, HEV RNAs were detected in 8 (19.5%). Of these, 6 samples were described in our previous study.[9]

Prevalence of HEV RNA in donors with elevated ALT in Japan

Thereafter, we studied a nationwide survey for HEV prevalence in Japanese blood donor samples with elevated ALT levels including levels of less than 500 IU per L, obtained from all Japan. Of 5,621,096 blood donations in 47 blood centers from April 2003 through March 2004, a total of 114,583 (2.0%) were disqualified because of elevated ALT levels of higher than 61 IU per L. Of these, 1389 donors (men vs. women, 5.5 vs. 1; age, 32 ± 11 years [mean ± SD]) showed elevated ALT level of higher than 200 IU per L. A total of 1062 donors with an ALT level of 61 to 199 IU per L were randomly collected from three blood centers as described.

The results are summarized in Table 2 and Fig. 2. Of 1389 donor samples with elevated ALT levels higher than 200 IU per L, 15 (1.1%) were HEV RNA–positive. Although the HEV-positive donor samples were found in any block of Japan, they tended to be more frequent in eastern Japan

TABLE 2. Prevalence of HEV RNA, IgM anti-HEV, and IgG anti-HEV among elevated ALT donors from April 2003 through March 2004 in Japan (total number of donors, 5,621,096)

Geographic blocks	ALT levels (61-199 IU/L)				ALT levels (200- IU/L)			
	Number of donors*	Number RNA-positive (%)	Number IgM-positive (%)	Number IgG-positive (%)	Number of donors†	Number RNA-positive (%)	Number IgM-positive (%)	Number IgG-positive (%)
Hokkaido	364	0 (0.0)	1 (0.3)	21 (5.8)	87	4 (4.6)	3 (3.4)	6 (6.9)
Miyagi	NA	NA	NA	NA	143	3 (2.1)	3 (2.1)	3 (2.1)
Tokyo	NA	NA	NA	NA	335	4 (1.2)	3 (0.9)	19 (5.7)
Aichi	NA	NA	NA	NA	223	1 (0.4)	2 (0.9)	6 (2.7)
Osaka	NA	NA	NA	NA	234	1 (0.4)	1 (0.4)	3 (1.3)
Okayama	345	0 (0.0)	0 (0.0)	7 (2.0)	188	1 (0.5)	1 (0.5)	1 (0.5)
Fukuoka	353	0 (0.0)	0 (0.0)	1 (0.3)	179	1 (0.6)	1 (0.6)	7 (3.9)
Total (95% CI)	1062	0 (0.0)	1 (0.1) (0.0-0.5)	29 (2.7) (1.8-3.9)	1389	15 (1.1) (0.6-1.8)	14 (1.0) (0.6-1.7)	45 (3.2) (2.4-4.3)

* Random sampling of donors with elevated ALT (61-199 IU/L) from three prefectures (Hokkaido, Hiroshima, and Fukuoka).
† All donor samples with elevated ALT levels of higher than 200 IU per L during this period.
CI = confidence interval; NA = not available.

(Hokkaido, Miyagi, and Tokyo; $p = 0.015$). No HEV RNA–positive sample was detected in 1062 donors with elevated ALT levels of 61 to 199 IU per L. The results indicate that HEV RNA–positive donors with elevated ALT levels higher than 200 IU per L were widely distributed over Japan and the prevalence was the highest in Hokkaido.

Antibodies against HEV in donors with elevated ALT levels in Japan

Of 1389 donor samples with elevated ALT levels higher than 200 IU per L, 14 samples (1.0%) were positive for the presence of IgM antibodies to HEV. Donors with IgM anti-HEV were also frequently found in eastern Japan ($p = 0.099$) and associated with positive HEV RNA (Table 2). Of 1062 donor samples with elevated ALT levels of 61 to 199 IU per L, only 1 sample was positive for the presence of IgM anti-HEV.

Of 1389 donor samples with elevated ALT levels higher than 200 IU per L, 45 samples (3.2%) were positive for the presence of IgG anti-HEV. Again, donors with IgG anti-HEV were more frequent in eastern Japan ($p = 0.003$) and not associated with HEV RNA–positive donors (Table 2). The frequency of IgG anti-HEV–positive donors appeared to be age-dependent, that is, from 0 percent of donors in their 10s to 12.5 percent of donors in their 60s (10s-30s vs. 40s-60s; $p < 0.0001$; Fig. 2). Of 1062 donor samples with elevated ALT levels of 61 to 199 IU per L, 29 samples (2.7%) were positive for the presence of IgG anti-HEV (Table 2). Again, the IgG anti-HEV–positive donors were more frequent in eastern Japan ($p < 0.0001$) and it appeared to be age-dependent (10s-30s vs. 40s-60s; $p = 0.001$, data not shown).

Analysis for HEV RNA–positive donors

We verified in detail the HEV RNA–positive samples obtained from two studies. Results of analyses for 8 (ALT ≥ 500 IU/L from Hokkaido) and 15 (ALT ≥ 200 IU/L from Japan) HEV RNA–positive donors are summarized in Table 3. The ensuing investigation revealed that all had no history of recent travel in HEV-endemic areas and remained asymptomatic despite of their elevated ALT levels. The concentration of HEV RNA varied from 1.9 to 7.5 log copies per mL. Of the 23 samples, 3 were seronegative, 2 were IgM anti-HEV–positive, 17 were IgM/IgG anti-HEV–positive, and 1 were IgG anti-HEV–positive samples. Twenty-three HEV RNA–positive samples were segregated into Genotype 3 ($n = 19$) and Genotype 4 ($n = 4$). These constituted 21 males and 2 females ages 25 to 62 years. Some of the 23 HEV RNA–positive donors were repeat donors. The results of the tests with samples from their other donations revealed that HEV RNA was detected in the previous donation in Donor 12 (HRC-HE12). The sample was negative for the presence of both IgM and IgG

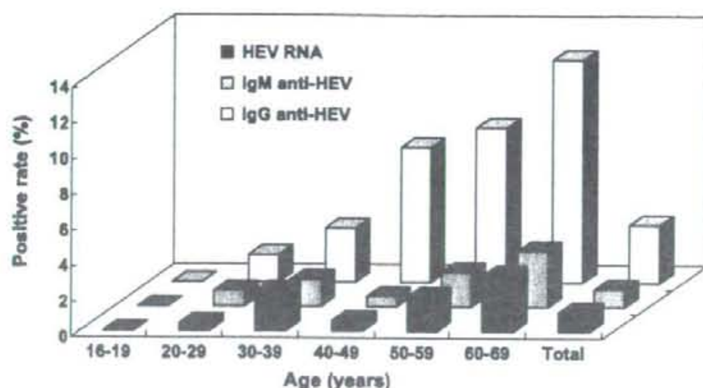

Fig. 2. Age-specific prevalence rates of HEV RNA (■), IgM anti-HEV (□), and IgG anti-HEV (□) in Japanese donors with elevated ALT levels of 200 IU per L and greater from April 2003 through March 2004. The total number of tested donors was 1389.

anti-HEV with normal ALT. The donated blood (whole blood) was not used for transfusion, because of the low volume of red cells. The plasma was in quarantine. Except for Donor 12, neither HEV RNA nor anti-HEV was detected in other donations.

When the 412-nucleotide ORF2 partial sequences of the HEV-positive 23 isolates were compared with those of reported HEV isolates from pigs or pig livers of Japan, all had a high nucleotide sequence identity of higher than 92.2 percent. More specifically, HRC-HE8 and JRC-HE5 had the highest nucleotide sequence identity, of 99.8 percent, with swJ11-4 and swJ19-1, respectively. Also, JRC-HE1, HRC-HE12, and HRC-HE3 had 99.3, 99.3, and 98.8 percent identities with swJ18-3, swJ13-1, and swJL145, respectively (Table 3).

DISCUSSION

The aim of this study was to investigate the prevalence of HEV among elevated ALT blood donors in Japan. The results of the primary study suggest that HEV was a major causative agent among blood donors with ALT levels higher than 500 IU per L in Hokkaido, since we demonstrated that HEV RNA was detected in 8 of 41 (19.5%) of the high ALT donor samples. Subsequently, a nationwide survey for HEV prevalence in blood donor samples with elevated ALT from all JRC revealed that 1.1 percent (n = 15) of donor samples with elevated ALT levels higher than 200 IU per L were positive for the presence of HEV RNA. No HEV RNA–positive samples were detected in donor samples with elevated ALT levels of 61 to 199 IU per L. Although the 15 HEV RNA–positive donors were widely distributed over Japan, they were frequently found in eastern Japan, especially in Hokkaido (4/15), Miyagi (3/15), and Tokyo (4/15).

It should be noted that in Hokkaido, 8 of the 41 donors with ALT levels of 500 IU per L or greater were positive for the presence of HEV, which is known to be transmitted by transfusion. Thus, as a result of performing HEV tests as the following study among 124 blood donors with ALT levels of 200 to 499 IU per L in Hokkaido, 1 donor (0.8%) was HEV RNA–positive (data not shown). Based on these results, in the subsequent study we expanded the area of investigation to nationwide and studied HEV prevalence in Japanese blood donor samples with elevated ALT including levels of less than 500 IU per L, obtained from all Japan. As for the geographical distribution of hepatitis E in Japan, it was reported that there was a higher prevalence of HEV-infected donors in the eastern part of Japan (Hokkaido, Miyagi, and Tokyo blocks).[15] We cannot clearly explain the reason why blood donors with HEV markers were more frequent in eastern than western Japan. Further studies with a larger number of donors including normal ALT levels will be necessary to draw a definitive conclusion.

Twenty-three HEV RNA–positive samples were divided into Genotype 3 (n = 19) and Genotype 4 (n = 4). Because it is commonly assumed that blood donors are healthy adults, most of those HEV-positive donors appeared to be asymptomatic. Since the isolates of acute hepatitis E patient samples were predominantly Genotype 4 in Japan,[19] the genotypes may play an important role in clinical progression of HEV infection. HEV-positive donors with ALT levels higher than 500 IU per L appeared to be asymptomatic and their ALT elevation was transient (unpublished observation).

In this study, the routes of HEV transmission of infected donors are not clear. The HEV RNA–positive donors had no history of recent travel abroad in areas where HEV is hyperendemic. Yazaki and his colleagues[4] reported that of the 363 packages of raw pig liver sold in grocery stores as food in Hokkaido, 7 (1.9%) packages had detectable HEV RNA. In this study, some isolates from the HEV RNA–positive donor samples showed close sequence homology with the isolates from pigs in Japan, suggesting that HEV transmission may be associated with the consumption of undercooked or inadequately cooked pig meat. Emerson and colleagues[20] reported that some HEV would most likely survive the internal temperatures of rare-cooked meat. When the 412-nucleotide ORF2 partial sequences of the 23 HEV RNA–positive donor isolates were compared with those of reported HEV isolates from pigs or pig livers of Japan, at least 9 isolates (39%) showed close sequence homology (98.5%-99.8%) with the

TABLE 3. Profile of HEV RNA–positive donors

Donor*	Geographic blocks	Date of donation	Age (years)	Sex	ALT (IU/L)	HEV RNA (log copies/mL)	Anti-HEV IgM	Anti-HEV IgG	HEV genotype	Strain	HEV strain with the highest homology among the known swine isolates [Accession No.] (%)†		
1	Hokkaido	Dec. 2000	29	M	767	5.6	+	+	4	HRC-HE1	swJL145‡	[AB105902]	(98.5)§
2	Hokkaido	Mar. 2001	30	M	506	5.0	+	+	3	HRC-HE2	swJHR1-1	[AB194528]	(93.9)
3	Hokkaido	Apr. 2001	40	M	1,470	6.9	+	+	4	HRC-HE3	swJL145‡	[AB105902]	(98.8)§
4	Hokkaido	Jul. 2001	47	M	713	5.1	+	+	3	HRC-HE4	swJTT1-1	[AB194526]	(93.4)
5	Hokkaido	Oct. 2001	62	M	2,080	6.3	+	+	3	HRC-HE9	swJL234‡	[AB105903]	(98.5)§
6	Hokkaido	Oct. 2001	39	M	641	5.1	+	+	3	HRC-HE5	swJL234‡	[AB105903]	(98.5)§
7	Hokkaido	Nov. 2001	48	M	740	3.6	+	+	4	HRC-HE6	swJL145‡	[AB105902]	(98.5)§
8	Hokkaido	Feb. 2003	39	F	578	6.2	–	+	3	HRC-HE7	swJL234‡	[AB105903]	(96.1)
9	Hokkaido	Jul. 2003	35	M	575	5.0	+	+	3	HRC-HE8	swJ11-4‡	[AB094243]	(99.8)§
10	Hokkaido	Oct. 2003	38	M	244	3.4	–	–	3	HRC-HE10	swJHK5-1‡	[AB194486]	(95.4)
11	Hokkaido	Nov. 2003	52	M	576	3.9	+	+	3	HRC-HE11	swJL234‡	[AB105903]	(96.1)
12	Hokkaido	Jan. 2004	38	M	793	5.9	+	+	4	HRC-HE12	swJ13-1‡	[AB094254]	(99.3)§
13	Miyagi	Dec. 2003	39	M	470	5.4	+	+	3	JRC-HE4	swJ24-1	[AB094306]	(92.5)
14	Miyagi	May 2003	25	M	222	4.2	+	+	3	JRC-HE6	swJL234‡	[AB105903]	(95.1)
15	Miyagi	Jan. 2004	34	M	273	3.8	+	+	3	JRC-HE7	swJ2-1‡	[AB094207]	(92.7)
16	Tokyo	Mar. 2004	41	F	216	1.9	+	+	3	JRC-HE9	swJAK6-2	[AB194512]	(93.7)
17	Tokyo	Jun. 2003	34	M	211	3.1	+	+	3	JRC-HE5	swJ19-1	[AB094279]	(99.8)§
18	Tokyo	Nov. 2003	34	M	447	6.8	–	–	3	JRC-HE1	swJ18-3	[AB094277]	(99.3)§
19	Tokyo	Feb. 2004	36	M	328	5.2	+	–	3	JRC-HE10	swJC1990	[AB096756]	(92.7)
20	Aichi	Feb. 2004	62	M	281	3.9	+	+	3	JRC-HE11	swJSZ1-1	[AB194524]	(92.2)
21	Osaka	Mar. 2004	37	M	793	5.9	–	–	3	JRC-HE8	swJHR1-1	[AB194528]	(95.9)
22	Okayama	May 2003	29	M	554	5.3	+	+	3	JRC-HE2	swJIW4-1	[AB194496]	(92.7)
23	Fukuoka	Aug. 2003	57	M	398	7.5	+	–	3	JRC-HE3	swJHR1-1	[AB194528]	(93.4)

* HEV RNA–positive donors: samples from Donors 1 through 8 were obtained from the primary study (ALT ≥ 500 IU/L from Hokkaido) and Donors 9 through 23 from the secondary study (ALT ≥ 200 IU/L from all Japan).

† Nucleotide sequences were compared to the GenBank databases utilizing the BLAST program available at http://www.ncbi.nlm.nih.gov as of March 2008.

‡ Isolates from Hokkaido.

§ Identities of 412-nucleotide ORF2 sequences over 98.5 percent are indicated.

+ = positive; – = negative; M = male; F = female.

isolates from pigs or liver of pigs.[2,4] It should be noted that among 12 HEV RNA–positive donors from Hokkaido, 10 isolates (83%) showed high nucleotide homology (>95%) of 412-nucleotide sequences with the isolates from pigs or pig livers from Hokkaido. The results are consistent with the possibility that at least some of the HEV RNA–positive donors were infected through the zoonotic food-borne route. Similarly, Feagins and colleagues[21] recently reported that of the 127 packages of commercial pig livers purchased from local grocery stores in the United States, 14 (11.0%) tested positive for the presence of HEV RNA. The widespread distribution of HEV is being clarified in developed countries other than Japan.[22,23]

In this study, IgM anti-HEV–positive as well as HEV RNA–positive samples were also frequently found in eastern Japan. IgM anti-HEV is known as a marker of the early seroconversion period. ALT elevation is observed in the early/middle stage of the infection; that is, ALT elevation follows viremia and accompanies/precedes seroconversion.[24] Most (12/15) of the HEV RNA–positive donor samples were positive for the presence of IgM anti-HEV. Of the 15 IgM anti-HEV–positive samples, 14 showed elevated ALT levels higher than 200 IU per L.

Although there were no HEV RNA–positive samples and only one IgM anti-HEV–positive sample detected in donors with elevated ALT levels of 61 to 199 IU per L, 2.7 percent of them were positive for the presence of IgG anti-HEV, which was comparable to the positive rate (3.2%) of IgG anti-HEV–positive donors with elevated ALT levels higher than 200 IU per L. In contrast to IgM anti-HEV–positive donors, IgG anti-HEV–positive donors were not associated with positive HEV RNA. There are several reports from Japan that IgG anti-HEV–positive samples are not rare (1.9%-14.1%) in blood donors with normal ALT levels who are mostly HEV RNA–negative.[13,25,26] In the present report we observed that the number of IgG anti-HEV–positive samples increased with advancing age in both groups, that is, one with an ALT level higher than 200 IU per L and the other with ALT levels of 61 to 199 IU per L. The IgG anti-HEV appears to be present for a prolonged period after infection. Ijaz and his colleagues[27] reported HEV-infected patients with non–travel-associated disease were more likely to be older and tended to be male in England. They estimated that male sex is a risk factor for acquiring the non–travel-associated disease. Most (14/15) of our HEV RNA–positive donors were also male. Because high-ALT-level donors were male-dominant, it will be necessary to investigate whether HEV RNA–positive donors were also male-dominant in ALT-normal donors. We also observed in this report that the number of IgG anti-HEV–positive donors increased with advancing age. This suggests that high prevalence of IgG anti-HEV in older Japanese persons is the consequence of their increased exposure to HEV with time. Among donors with ALT levels of higher than 200 IU per L, positive rates of IgG anti-HEV and HEV RNA were dissociated in Fukuoka (IgG anti-HEV vs. HEV RNA, 3.9% vs. 0.6%) and Tokyo (5.7% vs. 1.2%), in contrast to those (6.9% vs. 4.6%) in Hokkaido. These observations suggest that HEV infection was once prevalent in Fukuoka and Tokyo, while it is now prevalent in Hokkaido. It will be essential to investigate HEV prevalence among blood donors with normal ALT levels in each area of Japan to clarify these points.

As to the donors with ALT levels higher than 500 IU per L, our preliminary study indicated that, besides HEV, other viruses (hepatitis A virus [HAV], Epstein-Barr virus [EBV], cytomegalovirus [CMV], and human parvovirus B19 [B19] were detectable in some of the 41 donors (data not shown). Among hepatitis-associated viruses, screening tests including nucleic acid testing (NAT) for HCV and HBV have been implemented in Japan. Although ALT testing may not be very effective in the early stage of infection or as a surrogate test for HBV or HCV infection, it may be an effective method for eliminating the other hepatitis viruses in transfusion blood, especially HEV, HAV, EBV, CMV, and B19, which could be eliminated from blood for transfusion by ALT testing. Although the distinct populations collected during different periods, HEV RNA was detected in 8 of 41 (19.5%), 1 of 124 (0.8%), and 0 of 364 (0.0%) among donors with high ALT levels of 500 or greater, 200 to 499, and 61 to 199 IU per L in Hokkaido, respectively. Therefore, it is assumed that HEV RNA–positive rate may be lower among the ALT-normal donors (ALT < 61 IU/L) and that elimination of blood with high ALT levels may be effective in reducing the risk of infection caused by HEV. HEV NAT screening has been implemented as a trial in Hokkaido, the highest HEV-prevalent area in Japan.

Further, elimination of blood donors with ALT levels of 500 IU per L or greater would be an effective tool to reduce the infection risks of not only HEV but also HAV, EBV, CMV, and B19. Although ALT testing appears effective in decreasing the risk for infection of HEV, there are some problems. First, ALT testing resulted in the loss of much of the donor blood, which might have been appropriate for transfusion. Approximately 2 percent of donated blood is disqualified owing to an elevated ALT level of greater than 60 IU per L in Japan. Ninety-eight percent of these donors had an ALT level of less than 200 IU per L. Furthermore, studies in the United States and Europe have confirmed that values of ALT in normal males are considerably higher than those in normal females so that a single cutoff value for ALT rejects a higher proportion of men than women.[28,29] Second, hepatitis viruses including HEV RNA were detected in ALT-normal donors. It has been reported that HEV RNA–positive samples were detected in volunteer donors with ALT levels of 61 IU per L.[13] In the near future, it is necessary to compare the virus-positive rates both in normal and in high-ALT donors and to reevaluate

a cutoff value of ALT after considering the balance of the benefits and costs.

Besides ALT testing, IgM anti-HEV screening may be effective to eliminate asymptomatic HEV RNA–positive donors in the middle stage of infection. Most of the HEV-positive samples with high ALT levels were also positive for the presence of IgM anti-HEV, although neither ALT test nor IgM anti-HEV will be effective to eliminate HEV-positive donors in the window period. Since the zoonotic food-borne route appears to be a major cause of HEV infection in Japan,[1-8] it is most important to halt the potential spread of HEV by disseminating information on the risk of eating viscera or vaccination of animals as reservoirs.

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Serological Evidence for Hepatitis E Virus Infection in Laboratory Monkeys and Pigs in Animal Facilities in Japan

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Abstract: *In laboratory animal facilities, monkeys and pigs are used for animal experiments, but the details of hepatitis E virus (HEV) infection in these animals are unknown. The risk of infection from laboratory animals to humans has become a concern; therefore, much attention should be paid to the handling of these animals during their care and use, including surgical procedures performed on infected animals. In this connection, serum samples collected from 916 monkeys and 77 pigs kept in 23 animal facilities belonging to the Japanese Association of Laboratory Animal Facilities of National University Corporations (JALAN) and the Japanese Association of Laboratory Animal Facilities of Public and Private Universities (JALAP) in Japan were examined for the purpose of detecting antibodies to HEV and HEV RNA by using ELISA and RT-PCR, respectively. One hundred and seven serum samples of 916 (11.7%) monkeys were positive for anti-HEV IgG, and 7 and 17 serum samples of 916*

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(0.8% and 5.3%) monkeys were positive for anti-HEV IgM and IgA, respectively. Thirty-six samples from 62 (58.1%) farm pigs were positive for anti-HEV IgG, whereas all samples tested from miniature pigs were negative (0/15, 0%). Seven samples from 62 (9.1%) farm pigs and 7 samples from 916 (0.8%) monkeys were positive for IgM antibody, but these HEV-IgM antibody positive serum samples were HEV-RNA negative by RT-PCR. The IgM antibody positive rate (9.1%) of farm pigs was much higher than that of monkeys (0.8%). These results suggest the relative levels of risk of HEV infection from these animals to animal handlers and researchers who work with them in laboratory animal facilities.
Key words: *anti-HEV antibodies, experimental animals, hepatitis E virus, monkeys, pigs*

Introduction

Hepatitis E virus (HEV) is one of the most important causes of acute hepatitis in many developing countries in Asia [32], the Middle East and North Africa [22], and was recently classified as the sole member of the genus Hepevirus in the family Hepeviridae [5]. Hepatitis E was first recognized when a large water-borne hepatitis outbreak occurred in India in 1955, in which the antibody-positive rate of hepatitis A virus was extremely high in all age groups [9]. Because HEV is transmitted via an oral-fecal route, contaminated drinking water and food are the primary source of this infection. Although hepatitis E is self-limiting and neither chronic nor persistent infection is observed in the adult population in general, a high mortality rate of 15–20% is reported in pregnant women [12].

HEV is a non-enveloped, single-stranded, positive-sense RNA virus [30]. By phylogenetic analysis, at least four major genotypes of HEV have been identified [25]. Genotype 1 (G1) HEV was isolated in Asia and Africa [22, 11], genotype 2 (G2) in Mexico [34], and genotypes 3 (G3) and 4 (G4) in the United States, European countries, China, Japan, and Vietnam [7, 20, 24, 27–29, 35]. These viruses are thought to comprise a single serotype [22].

Although most hepatitis E cases in developed countries were thought to have been imported from developing countries, recent studies have revealed that hepatitis E has occurred in patients who had never been abroad [24, 27]. The findings that genetically similar G3 and G4 HEVs have been isolated from pigs [21, 23], deer and wild boars [15, 20, 31], and that serum antibodies to HEV were detected in a variety of animals including pigs [29], deer, wild boars, wild rats [10], dogs, cats, cows [18, 26], and monkeys [1, 2, 6, 8, 33], suggest that hepatitis E is a zoonosis. Also, hepatitis E virus antibody prevalence among persons who work with swine [4] and among those who live in the rural, southern part of the People's Republic of China [13] has been reported. Recently, direct evidence of G3 HEV transmission from deer and wild boar meats to humans was clearly provided in Japan, suggesting that wild animals are the zoonotic reservoir of HEV in Japan [15, 31]; transmission to humans from pig visceral organs [36], and following surgical training conducted on pigs [3] has also been suspected.

In laboratory animal facilities in Japan, wild Japanese monkeys and pigs from farms are used as experimental animals. Since HEV infection is considered a zoonosis, it is important to pay attention to the possibility of infection in these animals. In this connection, a survey was done to detect HEV infection in monkeys and pigs that were used for animal experimentation. To find HEV infection in laboratory animals, and to examine the HEV infection rate, we performed assays for the detection of anti-HEV antibodies and HEV RNA from monkey sera and pig sera collected from animal facilities belonging to the Japanese Association of Laboratory Animal Facilities of National University Corporations (JALAN) and the Japanese Association of Laboratory Animal Facilities of Public and Private Universities (JALAP) in 2005. Anti-HEV IgG was found in 11.7% of monkeys and 48.6% of pigs that were used for animal experiments, suggesting that animal handlers and researchers should pay attention to HEV infection in laboratory animals. It is considered desirable to use pigs and monkeys that are negative for HEV antibody as often as possible.

Materials and Methods

Monkey and pig sera

Sera (including plasma) were collected from monkeys and pigs that had been used for animal experiments in 1998 and 2005 in 23 animal facilities belonging to JALAN and JALAP. Nine hundred and sixteen samples from monkeys (20 facilities) and 77 samples from pigs (12 facilities) were tested. The pigs included 62 farm pigs and 15 miniature pigs purposely bred for animal experiments. These sera were stored at −30°C until testing.

Preparation of recombinant virus-like particles

A recombinant baculovirus, Ac5480/7126, harboring the G1 HEV capsid protein gene with III amino acids deleted at the N-terminal was constructed as described previously [14]. In brief, Tn5 cells (High Five™, Invitrogen, San Diego, CA) were infected with Ac5480/7126 at a multiplicity of infection of 10 and incubated at 26.5°C for 7 days. The intact cells and cell debris were removed from the culture medium, then the recombinant virus-like particles (VLPs) of 53 kDa were concentrated by centrifugation at 100,000 × g for 2 h in an SW28 rotor (Beckman Instruments, Inc., Fullerton, CA). The VLPs were further purified by isopycnic binding in CsCl gradient [16]. Recombinant baculoviruses that expressed N-terminal truncated capsid proteins of G3 and G4 HEV were similarly prepared, and the VLPs of 53 kDa were also prepared (Li *et al.* unpublished).

Detection of anti-HEV antibodies in monkeys and pigs

Flat-bottom, 96-well, polystyrene microplates (Immulon 2, Dynex Technologies, Inc., Chantilly, VA) were coated with the purified VLPs (1 μg/ml, 100 μl/well). The plates were incubated at 4°C overnight. Unbound VLPs were removed, and the wells were washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and then blocked at 37°C for 1 h with 200 μl of 5% skim milk (Difco Laboratories, Detroit, MI) in PBS-T. After the plates were washed 4 times with PBS-T, monkey serum or pig serum (100 μl/well) was added at a dilution of 1:200 in PBS-T containing 1% skim milk. The plates were incubated at 37°C for 1 h and then washed 4 times as described above. The wells were incubated with 100 μl of peroxidase-conjugated goat anti-human IgG (H+L) (1:5,000 dilution) or anti-swine IgG (H+L) (1:4,000 dilution) (KPL, Guildford, UK) or anti-human IgM (μ;) (1:1,000 dilution) or anti-swine IgM (μ) (1:2,000 dilution) (KPL, Guildford, UK) or anti-monkey IgA (1:1,000 dilution) (Alpha Diagnostic Intl., Inc., San Antonio, TX) in PBS-T containing 1% skim milk. The plates were incubated at 37°C for 1 h and washed 4 times with PBS-T. Next, 100 μl of the substrate orthophenylenediamine (Sigma Chemical Co., St. Louis, MO) and H_2O_2 were added to each well. The plates were incubated in a darkroom at room temperature for 30 min, and then 50 μl of 4 N H_2SO_4 was added to each well. After the plates had stood at room temperature for 10 min, the absorbance at 492 nm was measured.

Western blot assay

Approximately 1 μg of VLPs was separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in 50 mM Tris-HCL (pH 7.4) and 150 mM NaCl, and incubated with monkey serum (1:500 dilution), followed by HRP-goat anti-human IgG (H+L) (1:1,000 dilution). The membrane was treated with ECL detection reagent (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions, and the luminescence was recorded by exposure to film (Fuji Film, Tokyo, Japan).

Detection of HEV RNA by RT-PCR

Total RNA was extracted with RNA zol-LS reagent (Tel-test, Inc., Friendswood, TX) using 200 μl of the monkey serum and then resuspended in 20 μl of DNase-, RNase-, and proteinase-free water. Reverse transcription (RT) was performed at 42°C for 50 min followed by 70°C for 15 min, with 1 μl of the oligo (dT) primer, 1 μl of superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD), 0.5 μl of 0.1 M dithiothreitol, 4 μl of 5 × RT buffer, and 1 μl of 10 mM deoxynucleoside triphosphates. Two microliters of the resulting cDNA were amplified in a 50-μl sample for nested PCR with Ex Taq DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan) with an external sense primer HEV-F1 (5'-TAYCGHAAYCAAGGHTGGCG-3'; nucleotide (nt) residues 5903-5922 of G1 Myanmar strain, GenBank

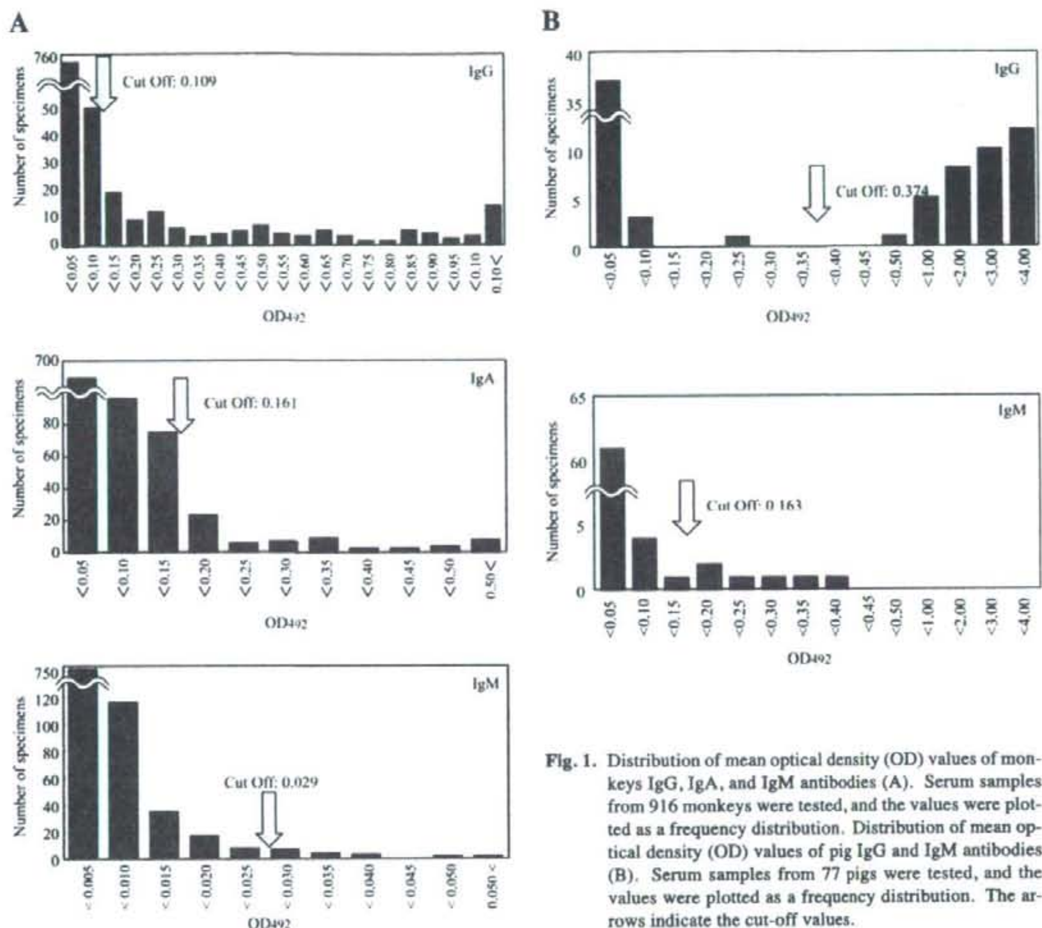

Fig. 1. Distribution of mean optical density (OD) values of monkeys IgG, IgA, and IgM antibodies (A). Serum samples from 916 monkeys were tested, and the values were plotted as a frequency distribution. Distribution of mean optical density (OD) values of pig IgG and IgM antibodies (B). Serum samples from 77 pigs were tested, and the values were plotted as a frequency distribution. The arrows indicate the cut-off values.

D10330) and an antisense primer HEV-R2 (5'-TGYTG-GTTRTCRTARTCCTG-3'; nt residues 6486-6467 of G1 Myanmar strain, GenBank D10330), using the GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA). Each cycle consisted of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and extension reaction at 7°C for 60 sec, followed by final extension at 72 °C for 7 min. The nested PCR was done with the internal sense primer HEV-F2 (5'-GGBGTBGCNGAGGAG-GAGGC-3'; nt residues 5939-5958) and the internal antisense primer HEV-R1 (5'-CGACGAAATYAAT-TCTGTCG-3'; nt residues 6316-6297) under the same conditions [15].

Results

Detection of anti-HEV IgG, IgM, and IgA in monkey sera and anti-HEV IgG and IgM in pig sera

The monkey serum samples were tested to detect anti-HEV IgG, IgM, and IgA at a dilution of 1:200 by ELISA. The distribution of the optical density (OD) values of monkey sera is shown in Fig. 1A. The OD values for IgM antibodies to HEV ranged from 0.000 to 0.136, and no serum samples with a titer of 200 had an OD value higher than 0.20. The OD values of anti-HEV IgG ranged from 0.000 to 1.926, and 83 sera whose titers ranged from 1:200 to 1:51,200 were higher than 0.20.

Table 1. OD values and antibody titers in monkey sera

Sera	OD values (1:200) (IgG/IgM)	Antibody titers (IgG/IgM)
No. 14	0.106/0.01	(<200)/(<200)
No. 15	0.017/0.002	(<200)/(<200)
No. 19	0.113/0.00	200/(<200)
No. 20	0.457/0.00	800/(<200)
No. 21	0.406/0.00	400/(<200)
No. 60	1.157/0.003	3,200/(<200)
No. 59	1.692/0.003	6,400/(<200)
No. 58	1.685/0.018	12,800/(<200)
No. 25	0.529/0.00	800/(<200)
No. 17	0.773/0.002	1,600/(<200)
No. 22	0.707/0.00	1,600/(<200)
No. 137	0.513/0.00	800/(<200)
No. 28	0.903/0.00	1,600/(<200)
No. 46	0.632/0.00	1,600/(<200)
No. 53	1.639/0.003	3,200/(<200)
No. 190	1.926/0.011	51,200/(<200)
No. 323	1.742/0.013	6,400/(<200)
No. 324	1.773/0.008	6,400/(<200)

OD values and antibody titers of IgG/IgM of several monkey samples are summarized in Table 1. The OD values of anti-HEV IgA ranged from 0.000 to 1.096, and 32 sera whose titers ranged from 1:200 to 1:6,400 were higher than 0.20 (data not shown).

The pig serum samples were also tested for the detection of anti-HEV IgG and IgM at a dilution of 1:200 by ELISA. The distribution of the OD values of pig sera is shown in Fig. 1B. The cut-off values, were determined in the same manner as the monkey sera, and are shown in the following section.

Specificity of IgG antibody in monkey sera

To determine whether the IgG antibody detected in monkey sera was specific to HEV, 7 serum samples were selected and examined by western blot assay (Table 1). The G1, G3, and G4 VLPs were separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The serum dilution of 1:500 was used for the assay. As shown in Fig. 2, strong bands with a molecular weight of 53 kDa corresponding to G1, G3, and G4 VLPs were detected in sample Nos. 58, 28, 137, and 22. The OD values of these sera were between 1.844, 0.347, 0.259, and 0.229 as determined by ELISA. Weak bands were detected in sample Nos. 21 and 20, whose OD values were 0.126 and 0.185, respectively, whereas no band was detected in sample No. 15, which had a low OD value of 0.017 as determined by ELISA. These results indicate that the anti-HEV-IgG detected in monkeys by ELISA was specific for HEV. In this survey, farm pigs were considered to be the reservoir of hepatitis E virus. As the IgG antibody detected in pig sera showed very high OD values without samples having OD values as low as those of the negative controls, we considered all positive samples to have antibodies specific to HEV. Therefore, determination by western blot assay was not conducted.

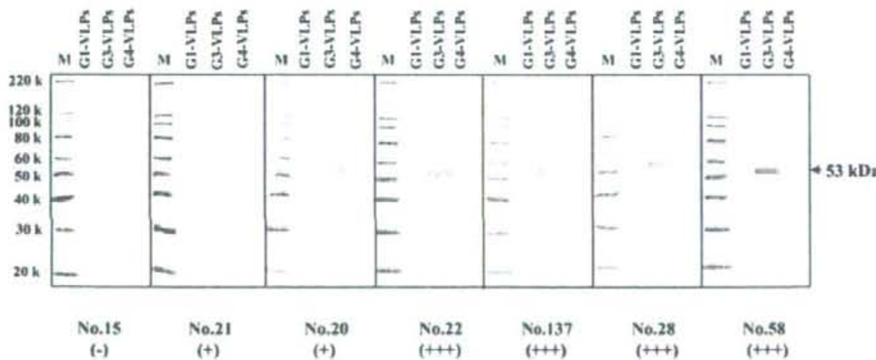

Fig. 2. Specificity of the IgG antibody determined by western blot assay. The genotype 1 (G1), G3, and G4 VLPs were used as the antigens; 7 monkeys' sera with different OD values were evaluated. Strong band (+++), weak band (+), and no band (–) by western blot assay are indicated. M: molecular weight marker.

Table 2. Results of antibody test for hepatitis E virus (HEV) in laboratory animals[a] (positive rates)

			IgG	IgM	IgA
Pigs[b]	Farm pigs	62	36 (58.1%)	4 (6.5%)	NT[c]
	Miniature pigs	15	0 (0%)	0 (0%)	NT
	Total	77	36 (46.8%)	4 (5.2%)	NT
Non-human primates[d]	Cynomolgus monkeys	115	9 (7.8%)	1 (0.9%)	4 (3.5%)
	Rhesus macaque	264	26 (9.8%)	0 (0%)	6 (6.1%)
	Japanese macaque	528	72 (13.6%)	6 (1.1%)	29 (5.5%)
	Taiwan macaque	6	0 (0%)	0 (0%)	0 (0%)
	Pigtail macaque	2	0 (0%)	0 (0%)	0 (0%)
	African green monkeys	1	0 (0%)	0 (0%)	0 (0%)
	Total	916	107 (11.7%)	7 (0.8%)	49 (5.3%)

[a]Samples with the OD values over (the mean of negative samples + 3SD) were classified as positive. [b]Sera (including plasma) of pigs that had been used and kept as laboratory animals. Samples were supplied during the period between August and December in 2005 (77 samples from 12 institutions). [c]Not tested. [d]Sera (including plasma) of non-human primates that had been used and kept as laboratory animals. Samples were supplied during the period between August and December in 2005 (627 samples from 19 institutions) and a part of the samples collected by the Japanese Association of Laboratory Animal Facilities of National University Corporations during the fiscal year of 1998 (289 samples).

Prevalence of anti-HEV IgG, IgM, and IgA in monkey and pig sera

The cut-off values of IgG, IgM, and IgA for the ELISA were determined with 833, 916, and 884 antibody-negative monkey serum samples, respectively. The OD values of anti-HEV IgG of these sera were between 0.000 and 0.193, and the mean value was 0.019 with SD of 0.03. Therefore, the cut-off value, the mean value +3SD, was calculated to be 0.109 (Fig. 1A). When this value was employed, the prevalence of anti-HEV IgG appeared to be 11.7% (107/916). The antibody-positive rate was 13.4% in females and 11.9% in males, however, the difference between the sexes was not statistically significant (P>0.05). The mean OD value of anti-HEV IgM for these 916 sera was 0.001 and that of SD was 0.009; thus, the cut-off value was calculated to be 0.029 ($0.001 + 3 \times 0.009$). Using this cut-off value, 7 monkey sera appeared positive for IgM antibody, and therefore the prevalence rate was 0.8% (7/916). The mean OD value of the 884 anti-HEV IgA-negative sera was 0.032 and that of SD was 0.043; thus, the cut-off value was calculated to be 0.161 ($0.032 + 3 \times 0.043$). Using this cut-off value, 17 monkey sera appeared positive for IgA antibody; therefore, the prevalence rate was 5.3% (49/916).

The cut-off value of IgG and IgM for the ELISA was determined with 40 and 70 antibody-negative pig serum samples, respectively. The OD values of anti-HEV IgG of these sera were between –0.662 and 0.248, and the mean value was –0.189 with SD 0.188. Therefore, the cut-off value, the mean value +3SD, was calculated to be 0.374 ($-0.189 + 3 \times 0.188$). When this value was employed, the prevalence of anti-HEV IgG appeared to be 46.8% (36/77). The mean OD value of anti-HEV IgM for these 70 antibody-negative pig sera was –0.049 and that of SD was 0.071; thus, the cut-off value was calculated to be 0.163 ($-0.049 + 3 \times 0.071$). Using this cut-off value, 7 pig sera appeared positive for IgM antibody; therefore, the prevalence rate was 9.1% (7/77).

The summary of the results of the positive rates of HEV antibody in the samples of laboratory animals are shown in Table 2. One hundred and seven serum samples of 916 (11.7%) monkeys were positive for anti-HEV IgG, and 7 and 49 serum samples of 916 (0.8% and 5.3%) monkeys were positive for anti-HEV IgM and IgA, respectively (Table 2).

Thirty-six sera of 77 (46.8%) pigs were positive for anti-HEV IgG. All positive samples were from the 62 farm pigs (36/62, 58.1%), whereas all samples tested from miniature pigs were negative (0/15, 0%). Four samples from farm pigs were positive for IgM antibody. The IgM antibody positive rate (5.2%) of pigs was much higher than that of monkeys (0.8%).

Detection of HEV RNA by RT-PCR

Four serum samples of 77 pigs were positive for HEV-IgM antibody as tested by RT-PCR for HEV RNA, but we were not able to amplify any HEV sequences in these samples.

Discussion

In the Biosafety Committee (Chair: Hiroshi Yamamoto, University of Toyama) of the Japanese Association of Laboratory Animal Facilities of National University Corporations (JALAN), various discussions regarding safety measures for humans have focused on the infection of laboratory animals in laboratory facilities, especially for zoonosis. As a part of this activity, a survey for B virus antibody in non-human primates was done and the results were published (*Exp. Anim.* 1998, 47: 199–202). In recent years, social concerns have been focused on hepatitis E transmitted from animals to humans. A survey on the prevalence of hepatitis E was conducted in laboratory animals kept in animal facilities belonging to the JALAN and the JALAP. Hepatitis E virus (HEV) is said to be a zoonosis and classified as Category IV by the Law Concerning Prevention of Infection of Infectious Diseases and Patients with Infectious Diseases in Japan.

In recent years, more pigs have been used as laboratory animals than in the past. Farm pigs, in which a high level of hepatitis E infection was reported, are used for animal experiments in medical and pharmaceutical research. Therefore, occupational health and safety measures for animal handlers and researchers are urgently needed. Non-human primates are also extremely important as laboratory animals for medical research. Chimpanzees, tamarins, African green monkeys, rhesus macaques and crab-eating macaques appear to be sensitive to HEV. Therefore, HEV infection was surveyed in monkeys and pigs kept in laboratory animal facilities. Based on the survey results, it is clear that persons working with laboratory animals should be well informed about HEV infection in laboratory animal facilities and safety measures to prevent this disease. For that purpose, the survey results are outlined and reported here.

An ELISA with recombinant HEV VLPs was used to detect anti-HEV IgG, IgM, and IgA antibodies in non-human primates and anti-HEV IgG and IgM in pigs. This assay was capable of detecting anti-HEV antibodies in human sera with high sensitivity and specificity [17]. The HEV specificity of the ELISA in monkey sera was confirmed by western blot assay in this survey. Through examination of pigs and monkeys kept in laboratory animal facilities, anti-HEV IgG-specific monkey antibody was detected. HEV antibody-positive rates were 60, 0, 13, 10, and 8 percent for farm pigs, miniature pigs, Japanese macaques, rhesus macaques, and crab-eating macaques, respectively. In general, farm pigs are thought to be highly contaminated with HEV. As the survey results indicate, researchers must pay due attention when using farm pigs for experiments. Generally, the possibility that antibody-positive animals, especially animals positive for IgG and IgA antibodies, carry or excrete HEV is fairly low. On the other hand, 7 farm pigs were confirmed to be positive for HEV IgM antibody. These animals may have been carrying HEV. Therefore, we thought it was necessary to conduct testing with PCR. However, we were not able to amplify any HEV sequences in these samples. Takahashi *et al.* suggested that pigs of 3 or 4 months age that were introduced from HEV positive farms sometimes carry the virus and excrete it in their feces [29]. Therefore, when using farm pigs for animal experiments, we should introduce 1–4 months pigs which are HEV negative to laboratory animal facilities. As for monkeys, our testing revealed that 107 serum samples of 916 animals tested were positive for anti-HEV IgG antibody, but there were no animals strongly positive for anti-HEV IgM antibody. There may have been no animals showing the symptom of viremia at the time of blood sampling. Therefore, the monkeys tested in this survey are considered not to be hazardous for use in experiments. It is notable that no infection with HEV was observed in miniature pigs purposely bred for experiments, though they were few in number in our sample. The safety of laboratory miniature pigs was confirmed with respect to HEV contamination.

Our study results confirm that past reports of pigs and monkeys being positive for HEV antibody in Japan [8, 29] were correct; however, no report of HEV infection attributable to pigs and monkeys that were used in experiments has been found so far. Three cases of HEV in humans were reported in the current Infectious Diseases

Weekly Report Digest (dated 14 April 2006), and one of them was attributable to consumption of pig liver. Uncooked meat of laboratory animals is never consumed by humans in Japan. The possibility remains, however, that HEV infection may occur due to accidental injection and injuries during experimental procedures and oral infection during animal handling. Furthermore, HEV antibody prevalence among persons who work with swine [4] and among those who live in the rural southern of the People's Republic of China [13] has been reported. Renou *et al.* and Colson *et al.* reported the possible zoonotic transmission of HEV through direct contact between a pet pig and its owner, and from surgical training on pigs, respectively, in France in 2007 [3, 23]. In swine, the duration of viremia after HEV infection is not very long [19]. IgM and IgG antibodies rapidly increase in the blood after infection, and infection risk decreases due to the increased antibody levels. In experiments using adult pigs, there is little possibility of encountering viremic animals; however, the infection pattern among animals is not clear, and the possibility of infection due to failures of experimental procedures cannot be denied. Therefore, animals from HEV-negative colonies should be used. By avoiding direct contact with blood and organs as well as paying close attention during washing procedures that may involve the abundant spraying of feces, it is possible to prevent infection from laboratory animals of humans.

It is very rare for animal handlers to become infected with HEV while taking care of animals positive for HEV antibody if they wear proper protective equipment against infection, such as masks *et cetera*, according to ordinary husbandry procedures. In large-scale laboratory animal rooms, animal handlers should use appropriate protective measures, because splashing occurs during some procedures, such as washing. When disposing of animals at the end of an experiment, if it has been confirmed that those animals were in the viremic state, workers must pay attention to the possibility of infection via blood and increase their safety precautions. Especially among pregnant women, HEV has a high infection rate and there is a high onset rate of fulminant hepatitis [12]. Khuroo *et al.* reported that the fulminant rate in pregnant women (22.2%) was higher than that in non-pregnant women (0%) and men (2.8%), and all cases of fulminant hepatitis in pregnancy occurred in patients who were in the last trimester [12]. In Japan the Labour Standards Law guarantees that a woman who is pregnant can take holidays for 6 weeks before birth and 8 weeks after childbirth, respectively, but holiday periods are not enough for preventing HEV infection. Therefore, animal handlers and researchers working with pigs and monkeys who are pregnant should be able to change their duties to other duties in the animal facilities during the last trimester. It is necessary for people at special risk to be exceptionally careful when they engage in work or experiments presenting possible exposure to HEV infection.

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