Table 1. Detection of antibodies to hantavirus by IFA among wild rodents in Honshu, Shikoku, kyushu, and Tsushima islands (2000-2003)

Rodent species	Seroprevalence at											Positive rate (%) to		
	Aomori	Niigata	Toyama	Yamanashi	Shizuoka	Shimane	Tokushima	Miyazaki	Tsushima	Kagoshima	Osaka	HTNV	SEOV	PUUV
A. speciosus[a]	0/34[b]	0/6	4/252	0/7	0/19	1/69	0/2	0/50	0/35	0/1	0/28	5/471 (1.1)	4/471 (0.84)	0/471 (0)
A. argentius	-	-	0/9	0/1	0/9	-	-	0/17	0/7	-	-	0/49 (0)	0/49 (0)	0/49 (0)
M. montebelli	-	-	0/11	-	-	-	-	-	-	-	-	0/11 (0)	0/11 (0)	0/11 (0)
E. smithi	-	-	0/11	-	-	-	-	-	-	-	-	0/11 (0)	0/11 (0)	0/11 (0)
M. minutus	-	-	-	-	-	-	-	-	0/1	-	-	0/1 (0)	0/1 (0)	0/1 (0)
M. musculus	-	-	-	-	-	-	-	-	0/4	-	-	0/4 (0)	0/4 (0)	0/4 (0)
R. norvegicus	-	-	2/39	-	-	-	-	-	-	-	-	2/39 (5.1)	2/39 (5.1)	0/39 (0)
C. dauricauni	-	-	-	-	-	-	-	0/1	-	-	-	0/1 (0)	0/1 (0)	0/1 (0)
U. tapeides	-	-	-	-	-	-	-	0/5	-	-	-	0/5 (0)	0/5 (0)	0/5 (0)
Total	0/34	0/6	6/358	0/14	0/28	1/69	0/2	0/56	0/45	0/1	0/28	7/502 (1.5)	6/502 (1.0)	0/502 (0)

a) Seropositive rodent species, the places where seropositive rodents found, and the number of seropositive rodents were shown in bold face.

b) Number of seropositive rodents/number of captured rodents

Table 2. Detection of antibodies to hantavirus (HTN or SEO or PUU) by IFA among rodents captured in field surveys in Hokkaido (2000-2003)

Rodent Species	Place of survey					Positive rate (%) to
	Nemuro	Kiritappu	Ebetsu	Tobetsu	Yufutsu	HTNV, SEOV, or PUUV
C. rufocanus	1/1[a]	0/8	3/8	1/125	0/11	5/153 (3.3)
C. rutilus	0/20	-	-	-	0/2	0/22 (0)
A. speciosus	-	-	0/1	0/1	0/4	0/6 (0)
A. argentius	-	-	0/1	-	0/9	0/10 (0)
A. peninsulae	-	-	-	-	0/4	0/4 (0)
R. norvegicus	-	-	-	0/4	-	0/4 (0)
S. caecutiens	-	-	-	-	0/3	0/3 (0)
S. gracillimus	-	-	-	-	0/11	0/11 (0)
S. unguiculatus	-	-	-	-	0/1	0/1 (0)
	-	-	-	-	-	
Total	1/21	0/8	3/10	1/130	0/45	5/214 (2.3)

a) Number of seropositive rodents/number of captured rodents

Table 3. Detection of antibodies to hantavirus (HTN or PUU or SEO) by IFA among rodents captured in ports and the Chitose Airport in Hokkaido (2000-2003)

Rodent species	Place of survey									Positive rate (%) to		
	Chitose[a]	Otaru	Hakodate	Rumoi	Hanasaki	Ishikari	Kushiro	Wakkani	Tomokomai	HTNV	SEOV	PUUV
C. rufocanus	1/39					0/5				2/44 (4.5)	0/44 (0)	2/44 (4.5)
A. speciosus	0/5									0/5 (0)	0/5 (0)	0/5 (0)
R. norvegicus	0/37	1/19	0/23	1/56	0/4	0/9	0/3	0/35	0/56	2/321 (0.62)	2/321 (0.62)	0/321 (0)
R. rattus		2/43	1/1							3/45 (6.7)	3/45 (6.7)	0/45 (0)
Total	1/76	3/62	1/24	1/56	0/4	0/14	0/3	0/35	0/56	7/415 (1.7)	5/415 (1.2)	2/415 (0.48)

a) Airport

Table 4. ELISA and WB for anti-hantavirus antibody positive blood donor found in the Self-defense Force in Hokkaido

No. of blood donors and positives (%)	Place	Donor	ELISA		WB		IFA		
			HTNV	PUUV	HTNV	PUUV	HTNV	SEOV	PUUV
1 207 (0.48%)	Chitose	#195	0.816	0.113	(+)	NT[a]	128	256	<32
		Control	0.023	0.018	(−)	(−)	<32	<32	<32

a) Not tested

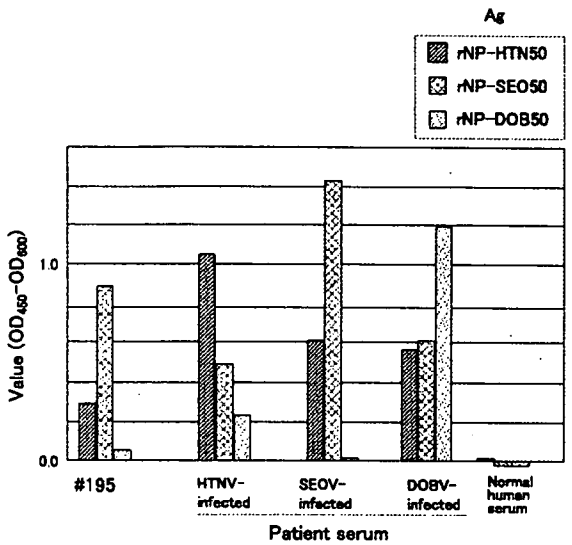

Fig. 2. Anti-hantavirus antibody detection by ELISA. IFA-positive human serum was incubated on a plate coated with recombinant hantavirus nucleocapsid proteins with serotype-specific antigenic sites. The plate was incubated with goat anti-human IgG conjugated with alkaline phosphatase. Optical density (OD) was measured at 405nm.

hantavirus infection in an at-risk population, we screened sera from the personnel of the Japan Ground Self-defense Force in Hokkaido. Of 207 blood samples examined, one sample was positive (0.48%) for anti-hantavirus antibody by IFA, with titers of 1 : 256 to SEOV and 1 : 128 to HTNV. To confirm this finding, we carried out ELISA and Western-blot analysis on the IFA-positive sample (Table 4). For ELISA, various recombinant nucleocapsid proteins (rNPs) were used as antigens to determine the type of infecting virus (Fig. 2). The IFA-positive serum reacted most strongly with an SEOV rNP (rNP-SEO50) in the ELISA test, and the reaction pattern was similar to that manifested by serum from a confirmed SEOV-infected patient (Fig. 2), indicating that the person might also have been infected with SEOV. Although this group of people has closer contact with *C. rufocanus* than does the general population, no antibodies to PUU-related virus were detected, suggesting that PUU-related virus carried by *C. rufocanus* in this area rarely infects humans.

Epidemiology of hantavirus infection in Far East Russia

A variety of hantaviruses have been identified in HFRS patients and rodents in China and Korea[16,29,31,37,38]. HFRS has been firmly established to be endemic to Far East Russia. However, the genetics of hantaviruses that are human pathogens have not been well defined. To determine the reason for the relatively low prevalence of hantavirus infection in humans in Japan, as compared to its surrounding countries, we conducted epidemiological surveys in Far East Russia, a highly endemic area of HFRS.

Epizootiological survey of rodents in Vladivostok

An epizootiological survey was carried out on a total of 122 rodents that were captured in a Vladivostok suburb. The results of serological screening of the rodent sera by IFA are shown in Table 5. The identified rodent species included *A. peninsulae* (70), *A. agrarius* (39), *C. rufocanus* (8), *Microtus fortis* (3), and *Tamias sibiricus* (2). Screening by IFA revealed that one *A. agrarius*

Table 5. Detection of antibodies to HTN and PUU viruses by IFA among rodents in Vladivostok

Rodent Species	No. of sera tested	Positives by IFA (%)	
		HTN	PUU
A. peninsulae	70	4 (5.7)	0
A. agrarius	39	1 (2.5)	0
C. rufocanus	8	1 (12.5)	1 (12.5)
M. fortis	3	0	0
T. sibiricus	2	0	0
Total	122	6 (4.9)	1 (0.8)

Table 6. Clinical history, serology, and virus detection of HFRS patients in Primorye region

Category	Patient No.	
	1	2
Age/Sex	53 years/Male	49 years/Male
Residence	Cavalerovo	Vostok
Onset of illness	May 22, 2000	June 9, 2000
Date of death	May 30, 2000	June 22, 2000
Cause of death	Gastrointestinal bleeding	Acute renal failure
IFA antibody to		
HTN 76-118	512	1024
SR 11	128	1024
PUU	32	32
PCR		
Lung	+	−
Liver	−	−
Kidney	+	−
Spleen	NA*	+
Brain	NA	−

(2.5%), four *A. peninsulae* (5.7%), and one *C. rufocanus* (12.5%) individual had antibodies to HTN and/or PUU viruses. Lung tissues from seropositive *A. peninsulae* were subjected to RT-PCR to amplify the virus genomes. Two (#61 and #63) of the four rodents that had high anti-HTNV IFA titers (1 : 256 and 1 : 512) were positive by PCR for both the S and M segments of hantavirus.

Clinicopathology of HFRS patients

We obtained the clinical histories of two fatal cases of HFRS that occurred in the Primorye region (Table 6). These patients, who lived in villages 400 and 600 km from Vladivostok, died 8-13 days after the onset of disease from gastrointestinal bleeding and acute renal failure. Serological screening showed that both patients were positive for hantavirus antibodies. Antibody titers to HTN and SEO viruses were apparently higher than to PUU virus. Lung, liver, kidney, spleen, and brain tissues from these HFRS patients were used for RT-PCR analysis, which showed that the lung and kidney tissues of patient No. 1 and the spleen tissue of patient No. 2 were positive for the hantaviral M segment.

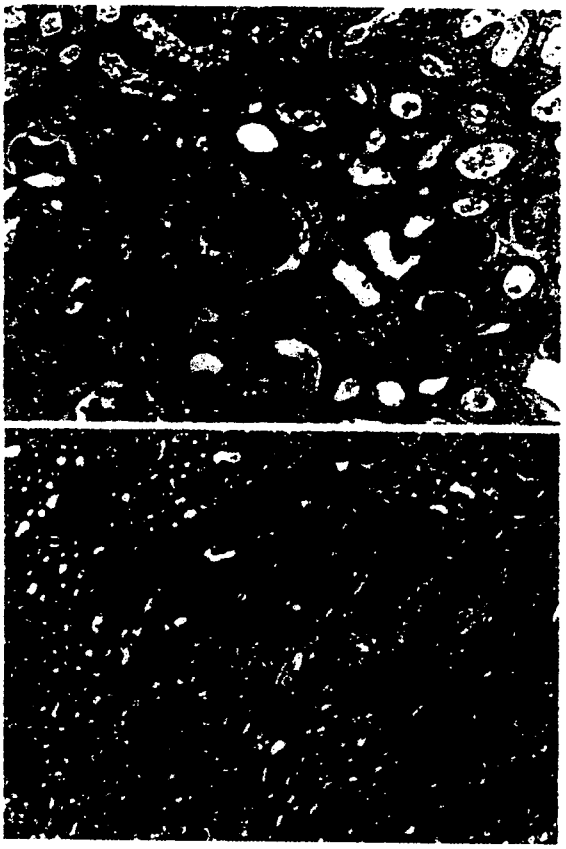

Fig. 3. Histopathological changes in kidney tissue from an HFRS patient in the Primorye region. The histological changes include interstitial edema with mild infiltration of mononuclear cells (small arrow) and the degeneration of renal tubules (large arrow) in the cortex. A: Proteinaceous casts and exudate (arrowhead) are seen in the luminae of renal tubules. Glomerular changes are inconspicuous. B:The most prominent change in the medulla is a well-defined necrotic lesion (asterisk).

Table 7. Comparison of nucleotide (2737-2969) and amino acid of M genome between those from *A. peninsulae*, HFRS patients in Primorye region, and other hantaviruses [a]

	Nucleotide identities %															
	SL/AP61	SL/AP63	AMR 1169	PRI/H1	PRI/H2	HB205	AMR/4313	HV114	A9	HTN76-115	Hojo	FE	NC167	DOB/SLO	SR-11	PUU
SL/AP61	-	99.5	95.8	96.1	99.2	94.8	94.3	88.7	85.7	84.4	82.7	82.7	79.3	79.7	79.7	86.3
SL/AP63	100.0	-	97.8	96.2	94.3	94.8	94.3	88.7	85.5	84.9	82.5	83.1	79.8	80.1	81.4	86.7
AMR/1169	98.8	98.2	-	96.5	95.7	95.6	95.6	86.6	86.2	84.9	85.1	81.4	79.7	80.1	79.5	86.3
PRI/H1	100.0	100.0	98.5	-	96.9	96.5	92.2	84.9	83.6	83.1	82.5	80.6	79.3	78.8	79.3	84.3
PRI/H2	99.7	98.7	98.5	98.7	-	94.8	94.3	85.7	85.3	84.0	82.3	81.4	78.9	79.2	79.8	86.4
HB205	100.0	100.0	98.2	100.0	98.7	-	91.5	83.6	85.1	85.3	84.9	80.6	77.1	79.5	77.1	86.7
AMR/4313	98.7	98.7	98.4	98.7	97.4	98.7	-	85.7	85.5	83.6	81.8	82.7	78.6	78.9	78.4	86.9
HV114	96.5	96.5	96.7	98.5	97.2	96.5	96.2	-	90.5	86.6	84.4	87.0	78.4	78.3	80.1	81.9
A9	97.5	96.5	96.5	96.5	96.2	98.5	98.2	98.9	-	86.2	84.0	87.5	78.0	78.4	81.5	80.6
HTN76-115	96.4	96.5	99.0	96.8	96.5	96.4	96.5	97.4	96.1	-	94.6	86.7	79.7	78.4	76.7	86.9
Hojo	96.8	96.5	96.0	96.8	96.5	96.8	96.5	97.4	96.1	100.0	-	87.9	78.9	79.3	76.7	91.5
FE	97.0	96.2	97.0	96.2	96.9	96.2	96.9	97.9	97.5	97.4	98.7	-	75.8	78.7	78.4	86.9
NC167	96.4	96.5	96.5	96.8	96.5	96.8	96.5	96.5	96.2	96.8	96.8	96.2	-	75.4	77.5	86.3
DOB/SLO	96.7	96.3	96.1	96.3	97.0	96.3	97.0	96.3	97.0	97.0	97.0	96.4	81.6	-	76.0	86.9
SR-11	96.1	96.1	96.3	96.1	96.8	96.1	96.8	96.1	96.8	96.8	96.8	96.1	80.3	80.6	-	86.6
PUU	83.2	83.7	83.2	83.2	81.9	83.4	81.9	82.9	82.5	81.9	81.6	83.2	81.6	79.4	81.2	-
	Amino acid identities %															

a) Values above the diagonal are nucleotide identities and those below the diagonal show amino acid identities

To examine the histopathological changes that occur in HFRS patients, we used light microscopy to examine sections of formalin-fixed lung, liver, kidney, spleen, and brain tissues from patient No. 2, who had died of acute renal failure (Fig. 3). We were able to detect pathological changes typical of severe HFRS[4,8,31,36], but the kidney was the only tissue that exhibited recognizable histopathological changes. The salient changes were interstitial edema with mild infiltration of mononuclear cells (small arrow) and degeneration of renal tubules (large arrow) in the cortex (Fig. 3A). Although proteinaceous casts and exudates were observed in the luminae of renal tubules (arrowhead), no glomerular changes were observed. In addition, a prominent, well-defined necrotic lesion (asterisk) was noted in the medulla (Fig. 3B). Monoclonal anti-HTNV antibodies failed to detect viral antigens in these specimens.

Sequence analysis of hantaviruses in the Primorye region

The 232-nt G2 region of the M segments of the viruses from two *A. peninsulae* specimens (#61 and #63) were amplified and sequenced. These viruses were designated as Solovey/AP61/1999 and Solovey/AP63/1999 (SL viruses) to indicate the name of the village closest to the survey point, the rodent species from which the sample was taken, and the year of the epizootiological survey. We also sequenced the M segments of the genetic lineages that were identified in the two HFRS patients from the Primorye region, designated as Primorye/H1/2000 and Primorye/H2/2000 (PRI viruses). When the M segments of the SL and PRI sequences were compared with those of other hantaviruses (Table 7), SL and PRI nucleotide sequences were 92.2-99.5% identical, and their amino acid sequences were identical or nearly so (98.7-100%).

We also compared the SL and PRI M segment sequences with those of the AMR genetic lineage, which were recently identified in HFRS patients and *A. peninsulae* in Far East Russia[17,40]. The nucleotide and amino acid identities between the SL/PRI and AMR lineages were 92.2-98.7% and 93.5-98.7%, respectively. Comparison of the M segment sequences of the SL-PRI-AMR lineages with

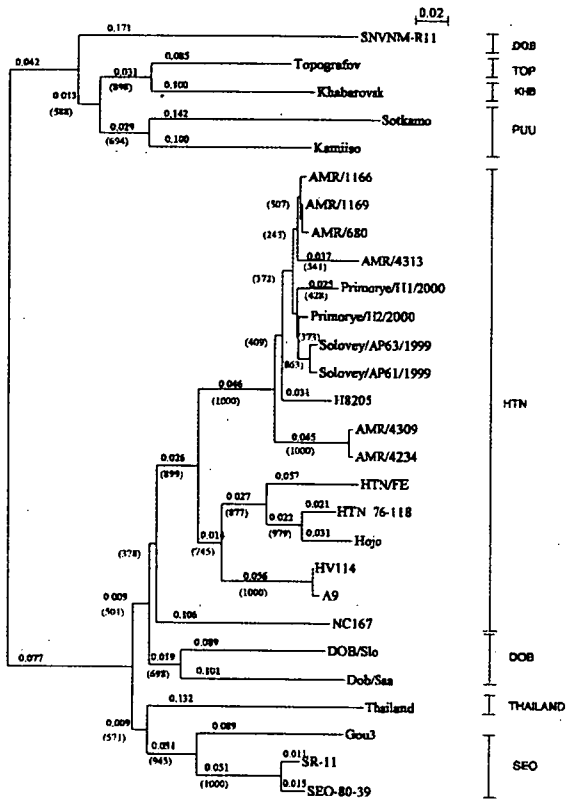

Fig. 4. Phylogenetic trees of the partial M segment (2736-2968 nt) of hantaviruses. The trees were constructed using the ClustalX (v. 1.81) program. The numbers above the branches are distances, and those in parentheses are bootstrap support values for 1000 replicates.

that of H8205, an isolate from an HFRS patient in China, revealed that their nucleotide and amino acid sequences were 91.3-95.6 and 94.8-100% identical, respectively. Lower levels of identity were found with the Hantaan, Seoul, Dobrava, and Puumala viruses. This high level of sequence identity among the SL, PRI, AMR, and H8205 sequences suggests that some patients in Far East Russia and China acquired the infection from the Korean field mouse (*A. peninsulae*). Our results also suggest that this genetic lineage is widely distributed throughout East Asia[3].

Phylogenetic analysis of the M segments of the Solovey, Primorye, and AMR sequences indicates that they form a common lineage with high bootstrap support values, regardless of viral origin, but separate from the prototype HTNV lineage (Fig. 4).

Table 8. IFA titers and PCR in acute HFRS patients in Khabarovsk

PatA No.	IFA titer to			PCR
	HTNV	SEOV	PUUV	M (G2)
1	16384	8192	16	-
2	1024	512	128	+
3	1024	512	<16	+
4	512	512	<16	-
5	256	128	<16	-
6	1024	512	64	-
7	1024	128	32	-
8	1024	128	<16	-
9	8192	8192	16	-
10	4096	512	<16	-
11	4096	256	<16	-
12	64	<16	<16	+
13	1024	512	<16	-
14	16384	2048	32	-
15	1024	512	32	-
16	64	<16	<16	-
17	512	512	<16	-
Control	<16	<16	<16	N.D.[a]

a) Not done

Serology of acute and convalescent HFRS patients by IFA

Sera from 17 acute and 32 convalescent patients who were clinically diagnosed with HFRS in the Khabarovsk region were examined for anti-hantavirus antibody against HTNV, SEOV, and PUUV antigens using IFA. Most of the samples from the 17 acute and 32 HFRS convalescent patients had IFA antibody titers to HTNV and SEOV ranging from 1 : 128 to 1 : 16384, while titers to PUUV ranged up to 1 : 128 (Table 8, 9). However, sera from two acute (PatA12 and PatA16) and one convalescent (PatC4) patient had low or no antibody titer to all viruses (the antibody titers were 1 : 64 or lower). In 10 acute and 25 convalescent patients, the difference in

Table 9. IFA titers in HFRS convalescents in Khabarovsk

ParC No.	IFA titer to		
	HTNV	SEOV	PUUV
1	1024	512	16
2	512	256	16
3	1024	1024	32
4	<16	<16	<16
5	8192	4096	128
6	2048	1024	64
7	1024	1024	32
8	4096	2048	64
9	4096	2048	32
10	4096	512	16
11	4096	1024	64
12	2048	1024	64
13	1024	1024	16
14	1024	512	32
15	4096	1024	32
16	1024	1024	<16
17	1024	1024	16
18	4096	1024	16
19	4096	1024	32
20	8192	2048	64
21	4096	2048	64
22	8192	2048	128
23	16384	8192	128
24	2048	4096	16
25	4096	8192	32
26	4096	4096	16
27	128	128	<16
28	2048	2048	16
29	8192	4096	32
30	8192	4096	32
31	256	256	32
32	1024	1024	16
Control	<16	<16	<16

HTNV and SEOV antibody titers was no more than two-fold.

Characterization of anti-hantavirus antibodies in acute patients by ELISA

The ELISA results for the acute patients are shown in Table 10. Sera from 12 of the 17 acute patients assayed by IgG ELISA using baculovirus-expressed rNPs[1,30,34] had higher optical densities (OD) for HTNV (range : 0.32 -2.07) than for PUUV (range : 0-0.04). Sera from 16 of 17 acute patients assayed by IgM ELISA using whole rNPs yielded higher ODs for HTNV (range : 0.71-2.40) than for PUUV (range : 0.01-0.14). Only PatA16 yielded a low OD value by both IgG and IgM ELISA (Table 10) and a low IFA titer (Table 8). Therefore, we did not consider PatA16 to be infected with hantavirus. Furthermore, we tried to distinguish the infecting virus by IgG ELISA using truncated rNPs (rNP50) (Table 10). All sera except PatA16 were positive for anti-HTNV antibodies. The ratio of rNP50s for

Table 10. Detection of anti hantavirus antibodies by ELISA in acute HFRS patients in Khabarovsk

Acute Pat No.	IgG (whole rNP)		IgM (whole rNP)		Infection	IgG (rNP50)			OD Ratio[b]	Serotype
	HTNV	PUUV	HTNV	PUUV		HTNV	SEOV	DOBV		
A1	2.07	0.01	0.96	0.02	+	1.70	0.05	-0.01	0.03	HTNV
A2	1.05	0.04	1.94	0.14	+	0.63	0.05	-0.01	0.08	HTNV
A3	0.57	0.00	1.71	0.02	+	0.57	-0.01	-0.01	0.01	HTNV
A4	0.49	0.01	1.19	0.02	+	0.49	0.01	0.00	0.02	HTNV
A5	0.31	0.01	0.97	0.04	+	0.30	0.06	-0.01	0.21	HTNV
A6	0.98	0.01	1.10	0.04	+	1.18	-0.01	-0.01	-0.01	HTNV
A7	0.15	0.00	1.04	0.03	+	0.45	0.07	0.00	0.15	HTNV
A8	0.30	0.05	0.71	0.02	+	0.42	0.03	0.00	0.04	HTNV
A9	1.91	0.02	1.48	0.03	+	1.18	0.17	0.02	0.15	HTNV
A10	0.82	0.00	1.75	0.03	+	0.48	0.07	0.01	0.14	HTNV
A11	0.75	0.01	2.34	0.08	+	0.75	0.14	0.01	0.19	HTNV
A12	0.05	0.00	1.19	0.01	+	0.23	0.00	-0.01	0.00	HTNV
A13	0.36	0.00	2.14	0.06	+	0.64	0.06	0.00	0.09	HTNV
A14	0.97	0.00	2.40	0.04	+	0.92	0.13	0.05	0.14	HTNV
A15	0.30	0.00	1.00	0.02	+	0.26	0.06	0.01	0.22	HTNV
A16	0.04	0.00	0.53	0.05	-	0.06	0.01	0.00	0.54	N.D.[a]
A17	0.32	0.00	0.85	0.02	+	0.45	0.02	-0.01	0.04	HTNV
Control[c]	0.00	0.00	0.08	0.02	-	0.00	0.00	0.00	N.D.	N.D.

a) Not determine

b) If the ratio of the OD of SEOV or DOBV rNP50 to that of to HTNV rNP50 was <0.7, the serum was deemed to be from an HTNV-infected human.

c) Nomal human sera

Table 11. Detection of anti hantavirus antibodies by IgG ELISA in HFRS convalescents in Khabarovsk

Convalescent	IgG ELISA (whole rNP)			IgG ELISA (rNP50)				
Pat No.	HTNV	PUUV	Infection	HTNV	SEOV	DOBV	OD ratio[b]	Serotype
C1	1.42	0.05	+	0.92	0.19	0.07	0.21	HTNV
C2	0.60	0.01	+	0.56	0.19	0.02	0.35	HTNV
C3	1.50	0.02	+	1.25	0.67	0.19	0.53	HTNV
C4	0.02	0.01	−	0.07	0.01	-0.01	0.00	ND[a]
C5	3.06	0.27	+	3.20	2.10	0.37	0.64	HTNV
C6	2.21	0.11	+	2.54	0.75	0.17	0.30	HTNV
C7	1.77	0.05	+	1.14	0.42	0.12	0.37	HTNV
C8	1.08	0.01	+	0.71	0.91	0.12	0.94	HTNV
C9	1.78	0.03	+	1.42	0.30	0.06	0.21	HTNV
C10	1.50	0.06	+	1.39	0.50	0.08	0.21	HTNV
C11	1.52	0.10	+	2.42	0.71	0.23	0.29	HTNV
C12	2.09	0.25	+	1.68	0.51	0.13	0.30	HTNV
C13	1.47	0.07	+	1.03	0.19	0.06	0.19	HTNV
C14	1.14	0.02	+	0.73	0.21	0.05	0.29	HTNV
C15	1.61	0.09	+	1.63	0.41	0.09	0.25	HTNV
C16	0.83	0.01	+	0.62	0.15	0.02	0.24	HTNV
C17	1.03	0.04	+	1.30	0.28	0.08	0.22	HTNV
C18	1.81	0.06	+	1.64	0.35	0.07	0.21	HTNV
C19	2.02	0.12	+	1.78	0.53	0.13	0.30	HTNV
C20	2.01	0.20	+	2.75	0.99	0.20	0.36	HTNV
C21	3.05	0.42	+	3.01	1.64	0.51	0.51	HTNV
C22	2.60	0.36	+	2.50	0.84	0.20	0.34	HTNV
C23	3.11	0.45	+	3.42	2.46	1.03	0.73	HTNV
C24	1.02	0.04	+	1.24	0.23	0.05	0.19	HTNV
C25	2.62	0.17	+	2.56	1.02	0.18	0.42	HTNV
C26	1.84	0.02	+	1.62	0.30	0.04	0.19	HTNV
C27	0.74	0.04	+	0.47	0.07	0.01	0.14	HTNV
C28	1.91	0.05	+	1.85	0.39	0.08	0.21	HTNV
C29	2.64	0.15	+	2.60	0.50	0.11	0.19	HTNV
C30	2.36	0.12	+	2.62	0.82	0.13	0.32	HTNV
C31	1.48	0.15	+	1.09	0.29	-0.02	0.20	HTNV
C32	1.74	0.07	+	1.48	0.38	0.10	0.27	HTNV
Control[c]	0.00	0.00	−	0.00	0.00	0.00	ND	ND

a) Not determined
b) If the ratio of the OD of SEOV or DOBV rNP50 to that of HTNV rNP50 was <0.7, the serum was deemed to be from an HTNV-infected human.
c) Nomal human sera

serum OD (second-highest value) to HTNV (highest value) was less than 0.7; therefore, all positive sera were deemed to be from patients infected with HTNV or HTN-related virus (Table 10).

Characterization of anti-hantavirus antibodies in convalescent patients by ELISA

The results of the IgG-ELISA with rNPs in convalescent patients are presented in Table 11. Most of the sera yielded higher ODs with HTNV rNPs (range: 0.6-3.11) than with PUUV rNPs (range: 0.01-0.45). Only PatC4 yielded low ODs for both rNPs (Table 11) and a low IFA titer (Table 9), suggesting that PatC4 had not been infected with hantavirus. Using rNP50 HTNV, SEOV, and DOBV antigens, most sera reacted strongly with rNP 50 HTNV antigen, as seen for the positive-control sera from a patient infected with HTNV. The SEOV/HTNV OD ratios of most sera were less than 0.7 (Table 11). Therefore, we conclude that 31 of these 32 convalescent cases were infected with HTNV or HTNV-related virus. Sera from convalescents who recovered from HFRS 10 to 30 years ago still

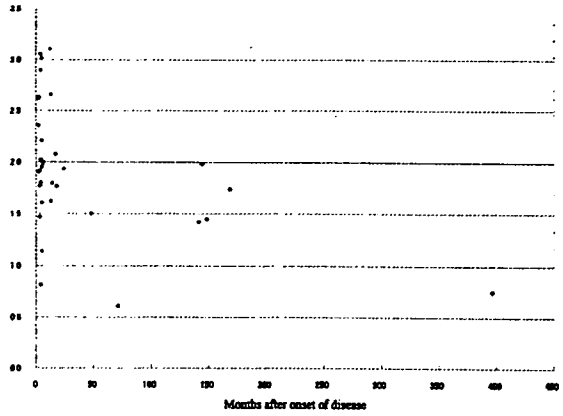

Fig. 5. Long-term persistence of anti-hantavirus IgG in convalescent HFRS patients in Khabarovsk.

yielded high ODs (Fig. 5).

Sequence analysis of hantavirus M genomes identified in acute patients

Hantavirus sequences were amplified from three acute patients (PatA2, 3, and 12) by RT-PCR. We sequenced partial M segments of the genome encoding the G2 region and compared them with those from other hantaviruses (Table 12). These sequences from the patients were found to be very closely related, with 97.4-98.3% nucleotide identities and 98.7-100.0% amino acid identities. The sequences were most closely related to FE3844, the Far East HTNV lineage[32], with 96.6-97.4% identity at the nucleotide level and 96.1-97.4% identity at the amino acid level. The nucleotide identities from high to low were: HTNV (88.8-89.7%), AMRV (81.0-82.7%), DOBV (76.6-77.9%), PUUV (65.3-66.7%), ANDV (65.2-66.5%), and SNV (59.1-60.3%).

Phylogenetic analysis of hantaviruses from patients with acute HFRS in the Khabarovsk region

The partial M genes were aligned using Clustal X. When a phylogenetic tree was constructed by the NJ method, PatA2, 3, 12, and FE3844 formed a phylogenetic cluster with high bootstrap support (Fig. 6). These sequences shared a common lineage with the prototype Hantaan virus (HTN76-118), but were distinct from the AMR genotype identified in Far East Russia and China (AMR7649,

Table 12. Nucleotide and amino acid identities of hantavirus sequences from acute HFRS patients in Khabarovsk with other viruses[a]

	Nucleotide identities (%)															
	PatA2	PatA3	PatA12	FE3844	HTN76-118	AMR7649	Solovey/AP63/1990	Primorye/H2/2000	H8266	Saaremaa/160V	SEO/SR-11	SEO/Vlad/S343	DOB/SLO	PUU/Kamiiso	AND/23	SN/NM RH
PatA2	-	97.4	97.4	96.6	89.7	82.3	82.3	81.4	81.0	79.4	77.9	77.5	76.9	66.3	66.1	59.1
PatA3	98.7	-	98.3	97.4	88.9	81.8	81.8	81.0	81.0	78.4	77.5	77.5	76.9	66.7	66.8	60.3
PatA12	100.0	98.7	-	97.4	89.7	82.7	82.7	81.8	81.0	77.9	77.1	76.6	76.5	66.8	66.2	59.5
FE3844	97.4	96.1	97.4	-	88.8	81.8	82.7	81.8	81.0	78.4	78.5	78.4	76.9	66.7	66.1	59.1
HTN76-118	100.0	98.7	100.0	97.4	-	84.4	84.4	84.4	85.7	79.2	77.1	76.2	75.8	62.6	64.3	58.1
AMR7649	93.5	92.2	93.5	96.9	93.5	-	96.7	97.0	94.4	75.9	77.9	77.1	76.9	66.2	62.5	60.2
Solovey/AP63/1990	93.5	92.2	93.5	96.9	93.5	99.4	-	97.8	94.4	76.3	81.0	80.1	79.7	63.9	62.5	57.0
Primorye/H2/2000	93.5	92.2	93.5	96.9	93.5	97.4	97.4	-	94.8	75.9	79.2	78.4	79.2	62.5	62.9	58.4
H8266	94.8	93.5	94.8	96.2	94.8	98.7	98.7	98.7	-	77.6	77.5	77.1	76.5	63.4	61.6	59.7
Saaremaa/160V	87.0	85.7	87.0	84.4	87.0	84.4	84.4	84.4	85.7	-	74.9	74.0	81.9	66.7	61.9	61.5
SEO/SR-11	81.8	81.8	81.8	83.1	81.8	81.8	81.8	81.8	83.1	77.9	-	94.8	75.2	62.9	64.4	58.7
SEO/Vlad/S343	81.8	83.1	81.8	83.1	81.8	81.8	81.8	81.8	82.1	77.9	96.7	-	76.6	63.3	65.8	58.9
DOB/SLO	87.0	85.7	87.0	84.4	87.0	87.0	87.0	87.0	88.3	90.9	80.5	80.5	-	61.6	61.9	59.3
PUU/Kamiiso	67.1	68.4	67.1	69.6	67.1	69.7	68.4	67.1	68.4	64.5	66.0	67.3	64.6	-	67.6	62.4
AND/23	65.8	67.1	65.8	65.8	65.8	61.0	59.7	58.4	59.7	63.2	58.4	59.7	64.5	68.8	-	75.9
SN/NM RH	61.5	65.8	64.5	61.9	64.5	58.4	57.1	55.8	57.1	56.6	53.2	54.5	65.2	65.8	81.8	-
	Amino acid identities (%)															

a) Values above the diagonal are nucleotide identities and those below the diagonal show amino acid identities

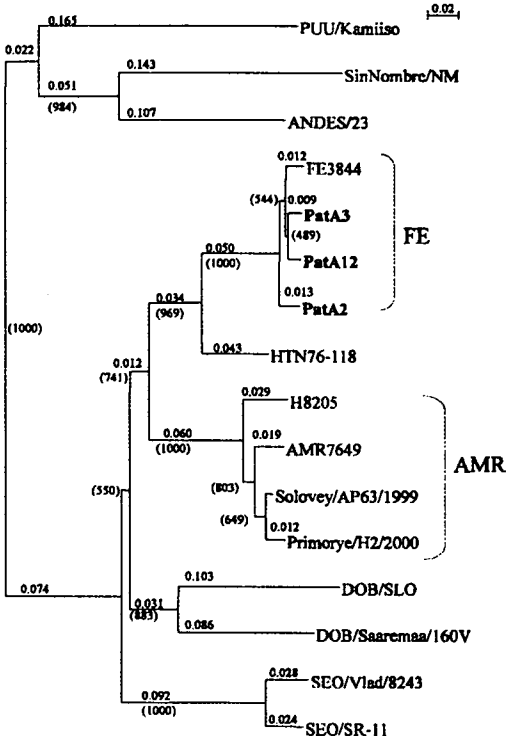

Fig. 6. Phylogenetic tree for the M segment of the hantavirus genome. The Clustal X program package was used to generate the phylogenetic trees using the NJ method with 1000 bootstrap replicates.

Solovey/AP63/1999, Primorye/H2/2000, and H 8205)[45,46]. The virus sequences from Khabarovsk patients fell in the same cluster as Far East virus, which was identified in HFRS patients in Far East Russia[39]. We did not identify the AMR genotype of HTNV infections in either acute or convalescent patients. Since the AMR genotype is genetically close to that of HTNV, the NP antigenicity of the two types may be similar. Therefore, we cannot exclude the possibility that some HFRS patients were infected with the AMR genotype. Antigenic characterization of the prototype HTNV, AMR and FE types is now in progress.

Although seropositive *C. rufocanus*, which carries PUUV-related virus in Japan[10], inhabit this region, no evidence of PUUV or PUUV-related virus infections was found in the acute or convalescent patients. An epidemiological survey to examine whether *C. rufocanus* carries PUUV-related virus in this region, as it does in Hokkaido, Japan, is planned.

Conclusion

Using epizootiological, clinical, pathological and sequencing studies, we identified a hantavirus carried by *A. peninsulae* as one of the causative agents of HFRS. We believe that this information may be helpful in preventing human infections not only in Far East Russia but also in eastern Asia. Since *A. peninsulae* is distributed over vast areas, including Far East Russia, China, Korea, and Japan, and hantaviruses closely related to the AMR genotype have been isolated from HFRS patients in China[37], considerable numbers of HFRS cases may be caused by the AMR genotype in these regions. Revealing the antigenicity of the AMR genotype glycoprotein, which is responsible for induction of neutralizing antibody and protective immunity, is very important. Since vaccine strains used in China and Korea are closely related to prototype HTNV, determining whether the antibody to HTNV can neutralize the AMR genotype is essential. Our recent cross-neutralization study revealed that the anti-sera against HTNV and AMRV had neutralizing antibody titers to the homologous virus that were ≧8 times higher than those to the heterologous virus. Therefore, vaccination with HTNV may be insufficient to prevent AMV infection.

Serological results indicate that FE genotype infection may be predominant in the Khabarovsk region. However, since *A. peninsulae* inhabit this region, AMR infections may also occur. Taken together, the AMR and FE genotypes are circulating in far eastern Russia, and both genotypes cause severe HFRS.

The animal reservoir of the FE genotype has not yet been determined, and *C. rufocanus* may carry a distinct hantavirus in Far East Russia. Therefore, further epizootiological studies are required to reveal the comprehensive ecology of hantaviruses in this area.

The result of this comparative epidemiological study of hantaviruses in Japan and Far East Russia suggests that the low prevalence of HFRS in Japan might be attributable to four principal factors : (1) no *A. agrarius*, and only a small number of *A. peninsulae*, the main reservoirs of HTNV and AMRV, inhabit Japan ; (2) infections caused by SEOV acquired from *R. norvegicus* and *R. rattus* may be mild and easily misdiagnosed ; (3) human infection by PUU-related virus from *C. rufocanus* occurs only rarely ; and (4) the prevalence of hantavirus infection in *A. speciosus* is low.

Hantavirus is one of the typical rodent-borne zoonotic agents. The pathogenicity of the virus largely depends on the virus type that is carried by the specific rodent host. Therefore, the reservoir rodent species of each virus type must be determined if anti-infective strategies are to be successful. Careful epidemiological study should be conducted to evaluate the hantavirus infection risk to the public in each local setting, since additional hantaviruses that are human pathogens may yet be discovered.

Acknowledgments

We acknowledge the invaluable contributions of the Japanese and Russian people, including scientists, support staff, and students, who supported this study. Without their collaboration, we could never have accomplished this study. We especially thank Kimiyuki Tsuchiya of the Tokyo University of Agriculture, Hitoshi Suzuki of Hokkaido University, and Masahiro Iwasa of Nippon University for providing rodent information. We appreciate the kind suggestions by Drs. Nobuo Hashimoto and Takeshi Kurata concerning epizootiological and epidemiological surveys. We also thank Mr. Michio Haseyama, Mr. Hiromi Nakai, Mr. Bisho Sakumoto, Mr. Takahara Noda of the Otaru Quarantine Office, and Ms Naomi Sakon of the Osaka Prefecturel Institute of Public Health for providing rodent materials. We appreciate the kind cooperation of Drs. Hiroyuki Wakiyama and Tatsuya Fujii of the Medical School of the Japan Ground Self-Defense Force during the field survey and collection of human sera.

This study was supported by the Grants-in-Aids for Scientific Research from Japan Society for the Promotion of Science. This study was also supported by the Grants from Program of Excellence for Zoonosis Control, Hokkaido University and and Ministry of Health, Labour and Welfare.

References

1) Araki, K., Yoshimatsu, K., Ogino, M., Ebihara, H., Lundkvist, A., Kariwa, H., Takashima, I. and Arikawa, J. 2001. Truncated hantavirus nucleocapsid proteins for serotyping Hantaan, Seoul, and Dobrava hantavirus infections. *J. Clin. Microbiol.*, 39 : 2397-2404.

2) Arikawa, J., Yoshimatsu, K. and Kariwa, H. 2001. Epidemiology and epizootiology of Hantavirus infection in Japan. *Jpn. J. Infect. Dis.*, 54 : 95-102.

3) Baek, L. J., Kariwa, H., Lokugamage, K., Yoshimatsu, K., Arikawa, J., Takashima, I., Kang, J. I., Moon, S. S., Chung, S. Y., Kim, E. J., Kang, H. J., Song, K. J., Klein, T. A., Yanagihara, R. and Song, J. W. 2006. Soochong virus : an antigenically and genetically distinct hantavirus isolated from *Apodemus peninsulae* in *Korea*. *J. Med. Virol.*, 78 : 290-297.

4) Bren, A. F., Pavlovcic, S. K., Koselj, M., Kovac, J., Kandus, A. and Kveder, R. 1996. Acute renal failure due to hemorrhagic fever with renal syndrome. *Ren. Fail.*, 18 : 635-638.
5) Clement, J., Heyman, P., Mckenna, P., Colson, P. and Avsic-zupan, T. 1997. The hantaviruses in Europe : from the bedside to the bench. *Emerg. Infect. Dis.*, 3 : 205-211.
6) Elliot, R. M. 1990. Molecular biology of the *Bunyaviridae*. *J. Gen. Virol.*, 71 : 501-522.
7) Golovljova, I., Brussjolander, K., Lindegren, G., Vene, S., Vasilenko, V., Plyusnin, A. and Laundkvist, A. 2002. Hantaviruses in Estonia. *J. Med. Virol.*, 68 : 589-598.
8) Grcevska, L., Polenakovic, M., Oncervski, A., Zografski, D. and Gligic, A. 1990. Different pathohistological presentations of acute renal involvement in Hantaan virus infection : Report of two cases. *Clin. Nephrol.*, 34 : 197-201.
9) Horling, J., Chizhikov, V., Lundkvist, A., Jonsson, M., Ivanov, L., Dekonenko, A., Niklasson, B., Dzagurova, T., Peters, C. J., Tkachenko, E. and Nichol, S. 1996. Khabarovsk virus : a phylogenetically and serologically distinct hantavirus isolated from *Microtus fortis* trapped in Far East Russia. *J. Gen. Virol.*, 77 : 687-694.
10) Kariwa, H., Yoshizumi, S., Arikawa, J., Yoshimatsu, K., Takahashi, T., Takashima, I. and Hashimoto, N. 1995. Evidence for the existence of Puumala-related virus among *Clethrionomys rufocanus* in Hokkaido, Japan. *Am. J. Trop. Med. Hyg.*, 53 : 222-227.
11) Kariwa, H., Yoshimatsu, K., Sawabe, J., Yokota, E., Arikawa, J., Takashima, I., Fukushima, H., Lundkvist, A., Shubin, F. N., Isachkova, L. M., Slonova, R. A., Leonova, G. N. and Hashimoto, N. 1999. Genetic diversities of hantaviruses among rodents in Hokkaido, Japan and Far East Russia. *Virus Res.*, 59 : 219-228.
12) Kariwa, H., Yoshimatsu, K., Araki, K., Murphy, M. E., Ebihara, H., Ogino, M., Mizutani, T., Arikawa, J., Chayama, K., Kumada, H. and Takashima, I. 2002. Detection of hantaviral antibodies among patients with hepatitis of unknown etiology in Japan. *Microbiol. Immunol.*, 44 : 357-362.
13) Kawamata, J., Yamanouchi, T., Dohmae, K., Miyamoto, H., Takahashi, M., Yamanishi, K., Kurata, T. and Lee, H. W. 1987. Control of laboratory-acquired hemorrhagic fever with renal syndrome (HFRS) in Japan. *Lab. Anim. Sci.*, 37 : 431-436.
14) Lee, H. W., Lee, P. W., Tamura, M., Tamura, T. and Okuno, Y. 1979. Etiological relation between Korean hemorrhagic fever and epidemic hemorrhagic fever in Japan. *Biken J.*, 22 : 41-45.
15) Lee, H. W., Lee, P. W., Baek, L. J. and Chu, Y. K. 1990. Geographical distribution of hemorrhagic fever with renal syndrome and hantaviruses. *Arch. Virol.*, Suppl. 1 : 5-18.
16) Liang, M., Li, D., Xiao, S. Y., Hang, C., Rossi, C. A. and Schmaljohn, C. S. 1994. Antigenic and molecular characterization of hantavirus isolates from China. *Virus Res.*, 31 : 219-233.
17) Lokugamage, K., Kariwa H., Hayasaka, D., Cui, B. Z., Iwasaki, T., Lokugamage, N., Ivanov, L. I., Volkov, V. I., Demenev, V. A., Slonova, R., Companets, G., Kushnaryova, T., Kurata, T., Maeda, K., Araki, K., Mizutani, T., Yohimatsu, K., Arikawa, J. and Takashima, I. 2002. Genetic characterization of Hantaviruses transmitted by the Korean field mouse (*Apodemus peninsulae*), Far East Russia. *Emerg. Infect. Dis.*, 8 : 768-776.
18) Lokugamage, K., Kariwa, H., Lokugamage, N., Miyamoto, H., Iwasa, M. A., Hagiya, T.,

Araki, K., Tachi, A., Mizutani, T., Yoshimatsu, K., Arikawa, J. and Takashima, I. 2004. Genetic and antigenic characterization of the Amur virus associated with hemorrhagic fever with renal syndrome. *Virus Res.*, 101 : 127-134.

19) Lundkvist, A. and Plyusnin, A. 2002. Molecular epidemiology of hantavirus infections. p. 351-384. In : *The Molecular Epidemiology of Human Viruses,* Leitner, T. (ed.). Kluwer Academic Publishers, Boston-Dordrecht.

20) Morii, M., Yoshimatsu, K., Arikawa, J., Zhou, G., Kariwa, H. and Takashima, I. 1998. Antigenic characterization of Hantaan and Seoul virus nucleocapsid proteins expressed by recombinant baculovirus : application of a truncated protein, lacking an antigenic region common to the two viruses, as a serotyping antigen. *J. Clin. Microbiol.*, 36 : 2514-2521.

21) Mustonen, J., Helin, H., Pietila, K., Brummer-Korvenkontio, M., Hedman, K., Vaheri, A., and Pasternack, A. 1994. Renal biopsy findings and clinicopathologic correlations in nephropathia epidemica. *Clin. Nephrol.*, 41 : 121-126.

22) Nemirov, K., Vaputahti, O., Lundkvist, A., Vasilenko, V., Golovljova, I., Plyushina, A., Niemimaa, J., Laakkonon, J., Vaheri, A. and Plyusnin, A. 1999. Isolation and characterization of Dobrava (Saaremaa) hantavirus carried by the striped field mouse (*Apodemis agrarius*) in Estonia. *J. Gen. Virol.*, 80 : 371-379.

23) Peters, C. J., Gary, L. S. and Levy, H. 1999. Spectrum of hantavirus infection : Hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. *Annu. Rev. Med.*, 50 : 531-545.

24) Plyusnin, A., Vapalahti, O. and Vaheri, A. 1996. Hantaviruses : genome structure, expression and evolution. *J. Gen. Virol.*, 77 : 2677-2687.

25) Plyusnin, A., Nemirov, K., Apekina, V., Plyusina, A., Lundkvist, A. and Vaheri, A. 1999. Dobrava (Saaremaa) hantavirus in Russia. *Lancet,* 353 : 207.

26) Polenakovic, M., Grcevska, L., Gerasimovska-Tanevska, V., Oncevski, A., Dzikova, S., Cakalaroski, K. and Masin, G. 1995. Hantaan virus infection with acute renal failure. *Artif. Organs,* 19 : 808-813.

27) Schmaljohn, C. S., Jening, G. B., Hay, J. and Dalrymple, J. M. 1986. Coding strategy of the S genome segment of Hantaan virus. *Virology,* 155 : 633-643.

28) Schmaljohn, C. S., Schmaljohn, A. L. and Dalrymple, J. M. 1987. Hantaan virus mRNA : coding strategy, nucleotide sequence, and gene order. *Virology,* 157 : 31-39.

29) Schmaljohn, C. S., Arikawa, J., Hasty, S. E., Rasmussen, L., Lee, H. W., Lee, P. W. and Dalrymple, J. M. 1988. Conservation of antigenic properties and sequences encoding the envelope proteins of prototype Hantaan virus and two virus isolates from Korean haemorrhagic fever patients. *J. Gen. Virol.*, 69 : 1949-1955.

30) Schmaljohn, C. and Hjelle, B. 1997. Hantaviruses : A global disease problem. *Emerg. Infect. Dis.*, 3 : 95-104.

31) Shi, X. H., Liang, M. F., Hang, C. S., Gan, S., McCaughey, C. and Eliott, R. M. 1998. Nucleotide sequence and phylogenetic analysis of the medium (M) genomic RNA segments of three hantaviruses isolated in China. *Virus Res.*, 56 : 69-76.

32) Song, G., Hang, C. S., Liao, H. X., Fu, J. L., Gao, G. Z., Qiu, H. L. and Zhang, Q. F. 1984. Antigenic difference between viral strains causing classical and mild types of epidemic hemorrhagic fever with renal syndrome in China. *J. Infect. Dis.*,

150 : 889-894.

33) Song, G. 1999. Epidemiological progress of hemorrhagic fever with renal syndrome in China. *Chin. Med. J.*, 112 : 472-477.

34) Sugiyama, K., Morita, C., Matsuura, Y., Shiga, S., Komatsu, T., Morikawa, S. and Kitamura, T. 1984. Isolation of a virus related to hemorrhagic fever with renal syndrome from urban rats in a nonendemic area. *J. Infect. Dis.*, 149 : 473.

35) Tamura, M. 1964. Occurrence of epidemic hemorrhagic fever in Osaka city : First cases found in Japan with characteristic feature of marked proteinuria. *Biken J.*, 7 : 79-94.

36) Tang, Y. W., Xu, Z. Y., Zhu, Z. Y. and Tsai, T. F. 1985. Isolation of haemorrhagic fever with renal syndrome virus from *Suncus murinus*, an insectivore. *Lancet*, 1 : 513-514.

37) Wang, H., Yoshimatsu, K., Ebihara, H., Ogino, M., Araki, K., Kariwa, H., Wang, Z., Luo, Z., Li, D., Hang, C. and Arikawa J. 2000. Genetic diversity of hantaviruses isolated in China and characterization of novel hantaviruses isolated from *Niviventer confucianus* and *Rattus rattus*. *Virology*, 278 : 332-345.

38) Xiao, S. Y., Liang, M. and Schmaljohn, C. S. 1993. Molecular and antigenic characterization of HV114, a hantavirus isolated from a patient with haemorrhagic fever with renal syndrome in China. *J. Gen. Virol.* 74 : 1657-1659.

39) Yashina, L. N., Patrushev, N. A., Ivanov, L. I., Slonova, R. A., Mishin, V. P., Kompanez, G. G., Zdanovskaya, N. I., Kuzinai, I. I., Safronov, P. F., Chizhi-kov, V. E., Schmaljohn, C. and Netesov, S. V. 2000. Genetic diversity of hantaviruses associated with hemorrhagic fever with renal syndrome in the Far East of Russia. *Virus Res.*, 70 : 31-44.

40) Yashina, L., Mishin, V., Zdanovskaya, N., Schmaljohn, C. and Ivanov, L. 2001. A newly discovered variant of a Hantavirus in *Apodemus peninsulae*, Far Eastern Russia. *Emerg. Infect. Dis.*, 7 : 912.

41) Yoshimatsu, K., Arikawa, J., Tamura, M., Yoshida, R., Lundkvist, A., Niklasson, B., Kariwa, H. and Azuma. I. 1996. Characterization of the nucleocapsid protein of Hantaan virus strain 76-118 using monoclonal antibodies. *J. Gen. Virol.*, 77 : 695-704.

Arch Virol (2007) 152: 1375–1381
DOI 10.1007/s00705-007-0965-6
Printed in The Netherlands

Archives of Virology

Brief Report

Prevalence of antibody to hepatitis E virus among wild sika deer, *Cervus nippon*, in Japan

Y. Matsuura[1], M. Suzuki[2], K. Yoshimatsu[1], J. Arikawa[1], I. Takashima[3], M. Yokoyama[4], H. Igota[5], K. Yamauchi[6], S. Ishida[7], D. Fukui[8], G. Bando[8], M. Kosuge[8], H. Tsunemitsu[9], C. Koshimoto[10], K. Sakae[11], M. Chikahira[12], S. Ogawa[13], T. Miyamura[13], N. Takeda[13], and T. C. Li[13]

[1] Institute for Animal Experimentation, Graduate School of Medicine, Hokkaido University, Sapporo, Japan
[2] Laboratory of Wildlife Biology, Department of Environmental Veterinary Science, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan
[3] Laboratory of Public Health, Department of Environmental Veterinary Science, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan
[4] Museum of Human and Nature Activities, Hyogo, Japan
[5] Nishiokoppe Wildlife Association, Hokkaido, Japan
[6] Research Institute for Environmental Sciences and Public Health of Iwate Prefecture, Morioka, Japan
[7] Enterovirology Division, Department of Microbiology, Hokkaido Institute of Public Health, Sapporo, Japan
[8] Asahikawa Zoological Park and Wildlife Conservation Center, Asahikawa, Japan
[9] Research Team for Viral Diseases, National Institute of Animal Health, Ibaragi, Japan
[10] Department of Bio-resources, Division of Biotechnology, Frontier Science Research Center, University of Miyazaki, Miyazaki, Japan
[11] Department of Microbiology, Aichi Prefectural Institute of Public Health, Nagoya, Japan
[12] Infectious Diseases Research Division, Hyogo Prefecture Institute of Public Heath and Environmental Science, Kobe, Japan
[13] Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

Received January 25, 2007; accepted February 22, 2007; published online April 13, 2007
© Springer-Verlag 2007

Summary

We examined 976 sika deer serum samples, 159 liver tissue samples and 88 stool samples collected from 16 prefectures in Japan, and performed ELISA and RT-PCR assays to detect antibodies to HEV and HEV RNA, respectively. Although 25 (2.6%) of 976 samples were positive for anti-HEV IgG, the antibody titers were very low. The OD values ranged between 0.018 and 0.486, forming a single distribution rather than a bimodal distribution, suggesting that the antibody detected in this study was not induced by HEV infection, or that deer have low sensitivity to HEV. HEV RNA was not detected in these samples, also suggesting that deer may not play a role as an HEV reservoir.

Author's address: Tian-Cheng Li, Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan.
e-mail: litc@nih.go.jp

*

Hepatitis E virus (HEV), the sole member of the genus *Hepevirus*, is the causative agent of type E

acute hepatitis in humans [3]. HEV does not have an envelope and is likely to have icosahedral symmetry. The genome is a positive-sense single-stranded polyadenylated RNA molecule, and the 5′ end is capped [11]. The genome of HEV contains three open reading frames, ORF1, ORF2, and ORF3. ORF1 encodes 1693 amino acids (aa) encompassing nonstructural proteins involved in viral replication. ORF2 encodes a 660-aa capsid protein. ORF3 encodes a 123- or 114-aa protein of unknown function [23, 28].

To date, at least four major genotypes of HEV have been identified by phylogenetic analyses. Genotype 1 (G1) HEV was isolated from Asia and Africa [16, 18], genotype 2 (G2) from Mexico [26], Namibia and Nigeria [2, 12], and genotypes 3 (G3) and 4 (G4) from the United States, European countries, China, Taiwan, Japan and Vietnam [4, 13, 17, 19, 27–29]. These viruses are thought to comprise a single serotype [16].

Transmission of human HEV occurs primarily by the fecal-oral route through contaminated water in developing countries [1, 5]. Since 1997, when the first animal strain of HEV was isolated from swine in the United States, there has been much indirect and direct evidence indicating that hepatitis E is a zoonosis and that humans appear to be at risk of infection with swine HEV by cross-species infection [13–15]. Recently, direct evidence of HEV transmission from wild boar (*Sus scrofa*) to humans was provided in Japan, suggesting that these animals are the main zoonotic reservoir of HEV in this country [9]. Indirect evidence of HEV transmission from swine to humans has also been accumulated [22, 30].

Because a case of HEV infection from sika deer meat was reported by Tei et al., sika deer have been considered a possible reservoir in Japan [24, 25]. However, there is only limited surveillance data of HEV infection in deer. In this study, we collected serum samples from wild deer and examined them for the presence of anti-HEV IgG by an antibody ELISA using recombinant virus-like particles (VLPs) as the antigen. We also attempted to detect HEV RNA in serum, stool, and liver samples from the wild deer by RT-PCR analysis.

Between 2003 and 2006, 866 serum samples were collected from wild deer captured in Hokkaido, Iwate, Tochigi, Chiba, Nagano, Aichi, Mie, Hyogo, Shimane, Hiroshima, Oita, Fukuoka, Kumamoto, Miyazaki, and Kagoshima prefectures, and 110 serum samples were collected in 1991–1993 from a deer farm, where the deer were introduced from the habitat at Miyagi prefecture (Fig. 1). In Hyogo Prefecture, an estimated age of 0–10 years was assigned by the tooth replacements and counting cementum annuli of the fist incisors [6]. A total of 88 stool samples were collected from deer captured in Hokkaido, Iwate, Tochigi, Chiba, Nagano, Mie, Hyogo, Hiroshima, Oita, Fukuoka, Kumamoto, Miyazaki, and Kagoshima from 2004 to 2006. They were resuspended in 10 mM phosphate-buffered saline (PBS) to prepare a 10% suspension, shaken at 4 °C for 1 h, and clarified by centrifugation at 10,000 × *g* for 20 min. A total of 159 deer liver tissue were collected from Hyogo (50), Iwate (11) and Hokkaido (98) from 2003 to 2006. The tissue was resuspended in lysis buffer (Qiagen, Inc.) and homogenized. All of the specimens were stored at −20 °C until use.

Serum anti-HEV IgG antibody was detected by ELISA by the method described previously with slight modification [8]. Briefly, a flat-bottom 96-well polystyrene microplate (Immulon 2; Dynex Technologies, Inc. Chantilly, VA) was coated with the purified VLPs (1 μg/ml, 100 μl/well) derived from the G1 Myanmar strain [7]. 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, deer serum (100 μl/well) was added in duplicate 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 rabbit anti-deer IgG (H + L) (1:1000 dilution) (KPL, Guildford, UK) in PBS-T containing 1% skim milk. The plates were incubated at 37 °C for 1 h and washed 4 times with PBS-T. Then, 100 μl of the substrate orthophenylenediamine (Sigma Chemical Co., St. Louis, MO) and H_2O_2 was added to each well. The plates were incubated in a dark room at room temperature for 30 min, then

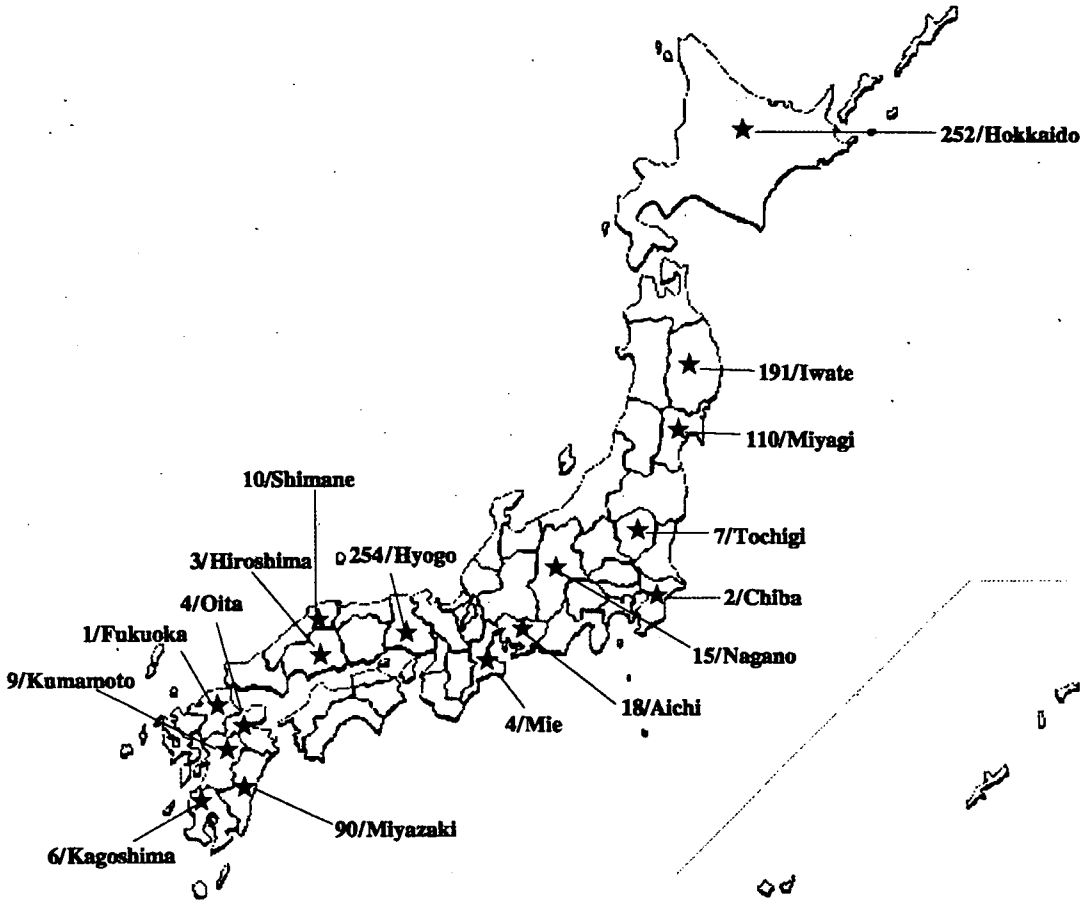

Fig. 1. Numbers and prefecture of captured wild sika deer

50 µl of 4N 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.

Anti-HEV IgG-positive serum was obtained from experimentally immunized captive sika deer that had been shown to be negative for HEV IgG by ELISA. The first and second immunizations were performed with purified VLPs (100 µg) in Freund's complete adjuvant by intramuscular injection at intervals of 2 week. After 2 weeks, the deer received booster injections of the same amount of VLPs in Freund's incomplete adjuvant. The deer was bled one week after the last booster injection. Pre-immunization serum was collected before administration and used as the negative control. Anti-HEV IgG-positive serum and pre-inoculation serum were stored at −30 °C. The anti-HEV IgG titer of the positive serum was 1:3,276,800.

Deer serum samples were tested for anti-HEV IgG at a dilution of 1:200 by ELISA. The distribution of the optical density (OD) values is shown in Fig. 2. The OD values of anti-HEV IgG ranged from 0.018 to 0.486 with the highest antibody titers being 1:400, and formed a single distribution. To determine whether the IgG antibody detected in deer sera was specific for HEV, the positive control serum and negative control serum, and the sera whose OD values were higher than 0.150 were selected and examined by Western blot assay. Approximately 1 µg of the VLPs derived from G1, G3, and G4 HEV was separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane.

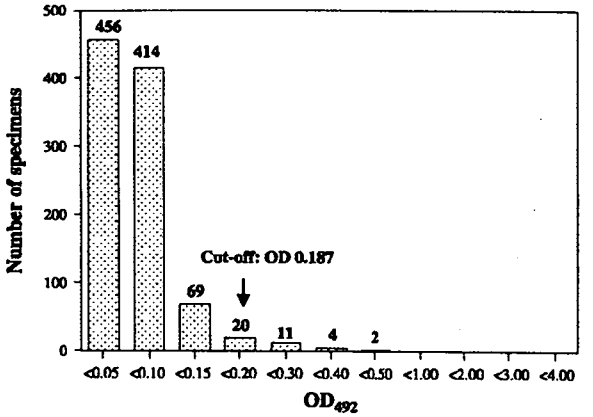

Fig. 2. Distribution of OD values of IgG antibodies. Serum samples from 976 deer were tested by ELISA. The arrows indicate the cutoff values

The membrane was then blocked with 5% skim milk in 50 mM Tris–HCl (pH 7.4) and 150 mM NaCl, and incubated with deer serum (1:200 dilution). Detection of deer IgG antibody was achieved by using phosphatase-labeled rabbit anti-deer IgG (H + L) (1:1000 dilution) (KPL, Gaithersburg, MD). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate P-toluidine were used as coloring agents (Bio-Rad Laboratories, Hercules, CA). As shown in Fig. 3, strong bands with a molecular weight of 53 k corresponding to the G1, G3 and G4 VLPs were detected with positive control sera. Weak bands were detected with Hyogo 0588, Hyogo 0409, and Miyagi 1, whose OD values were 0.486, 0.358, and 0.287, respectively, whereas no band was detected with Iwate 137, D0505, or the negative control serum, which had low OD values of 0.205, 0.152, and 0.051. These results indicated that the anti-HEV IgG detected in deer serum by ELISA was specific for HEV.

After eliminating 17 serum samples found to be positive by Western blot assay, 959 deer serum samples were used to evaluate the cutoff value of IgG. The OD values of these sera were between 0.018 and 0.248, and the mean value was 0.058 with a standard deviation (SD) of 0.043. Therefore, the cutoff value, the mean value + 3SD, was calculated to be 0.187 (Fig. 2). When this value was employed, the prevalence of anti-HEV IgG appeared to be

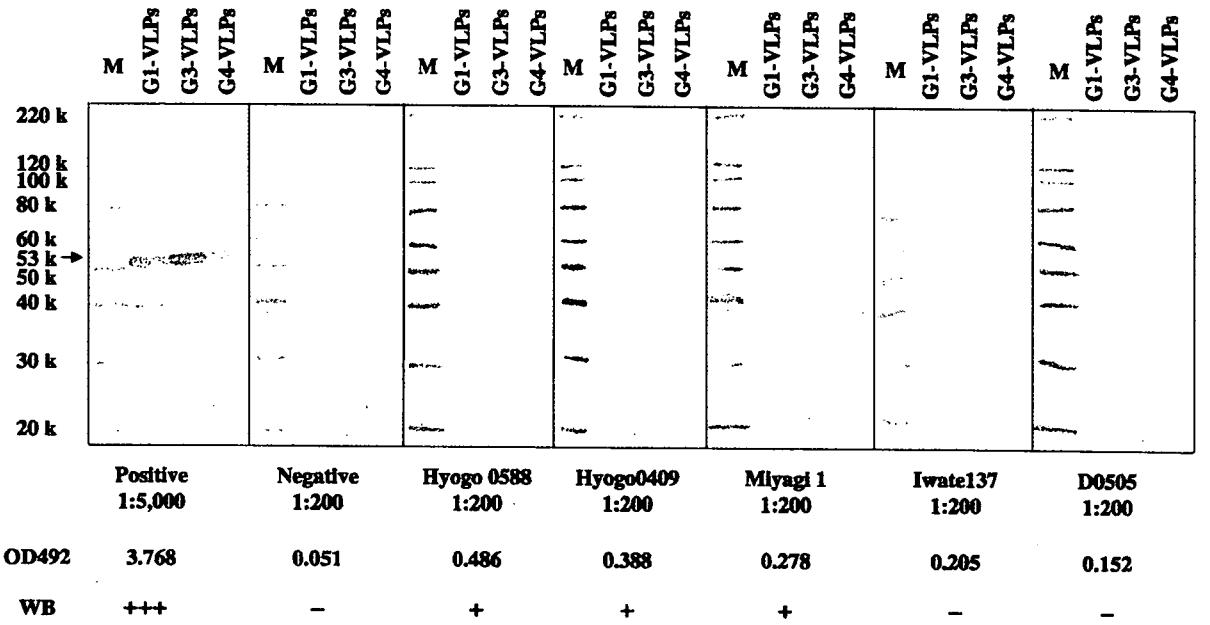

Fig. 3. Specificity of the IgG antibody determined by Western blot assay. The G1, G3, and G4 VLPs were used as the antigens, and 7 deer sera with different OD values were evaluated. The results of the Western blot assay are indicated as +++ (strong band), + (weak band), or – (no band). *M* Molecular weight marker

2.6% (25/976). The antibody-positive rate was 1.2% in Hokkaido, 2.2% in Miyazaki, 3.1% in Iwate, 3.1% in Hyogo, and 3.6% in Miyagi. The difference among these prevalence was not statistically significant ($P>0.05$).

Eighty-eight paired stool and serum samples from deer captured in Hokkaido (10), Iwate (23), Tochigi (7), Chiba (2), Nagano (5), Mie (4), Hyogo (8), Hiroshima (3), Oita (4), Fukuoka (1), Kumamoto (9), Miyazaki (6), and Kagoshima (6), 166 deer serum samples obtained in Aichi (18), Hyogo (28), Nagano (10), and Miyagi (110), and 159 deer liver tissue samples collected in Hyogo (50), Iwate (11) and Hokkaido (98) were tested by RT-PCR for HEV RNA. Total RNA was extracted with RNAzol-LS reagent (Invitrogen, Inc., Carlsbad, CA) using 200 μl of the deer serum, and 10% stool suspension Reverse transcription (RT) was performed at 42 °C for 50 min followed by 70 °C for 15 min in 20 μl reaction mixture containing 1 μl of Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Inc., Carlsbad, CA), 1 μl of the oligo (dT) primer, 1 μl of RNaseOUT™, 2 μl of 0.1 M dithiothreitol, 4 μl of 5×RT buffer, 1 μl of 10 mM deoxynucleoside triphosphates, 5 μl of RNA, and 5 μl of distilled water. Two microliters of the resulting cDNA was amplified in a 50 μl reaction mixture containing ExTaq DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan) with an external sense primer HEV-F1 (5′-GGBGTBGCNGAGGAGGAGGC-3′, nucleotide (nt) residues 5903–5922 of G1 Myanmar strain, D10330) and an external antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-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 an extension reaction at 72 °C for 60 sec followed by a final extension at 72 °C for 7 min. The nested PCR was done by using 2 μl of the first PCR product with an internal sense primer HEV-F2 (5′-TAYCGHAA YCAAGGHTGGCG-3′; nt residues 5939–5958) and an internal antisense primer HEV-R1 (5′-CGACGAAATYAATTCTG TCG-3′, nt residues 6316–6297) under the same conditions [9, 10]. Total RNA in deer liver was extracted from 100 mg of the tissue using an RNeasy Mini Kit (Qiagen, Inc.) and dissolved in 50 μl nuclease-free distilled water. The nested RT-PCR was carried out as described by Takahashi et al. [20]. However, we were not able to amplify any HEV sequences in these samples.

An ELISA with recombinant VLPs was used to detect anti-HEV IgG in sika deer in Japan. This assay was previously shown to be capable of detecting anti-HEV antibodies in human and mongoose sera with high sensitivity and specificity [8, 10]. To establish a system for detecting anti-HEV IgG in deer, we first prepared a positive control serum by immunizing deer with the G1 VLPs. After two doses of immunization, an antibody whose titer was as high as 1:3,276,800 was obtained. The specificity of this antibody was confirmed by Western blot assay, indicating that G1 VLPs was an excellent antigen to induce a strong immune response in deer.

In the present study, we tested a total of 976 deer serum samples for the presence of anti-HEV IgG antibody and made the following observations. First, the antibody prevalence was low in sika deer in Japan. Only 25 of 976 (2.6%) samples were positive for anti-HEV IgG by ELISA, which is lower than the prevalence in pigs (58%) and wild boars (44%), both of which are thought to be reservoirs of HEV in Japan [10, 21]. Second, the OD value and titer of anti-HEV IgG were low in deer. The highest OD value was 0.486 and the highest titer was 1:400. This observation is also different from that in pigs and wild boars, where the highest OD values were greater than 3.000 and the titers were greater than 1:51200. Third, the distribution of OD values indicated that only one peak was less than 0.486. The bimodal distribution observed in pigs and wild boars was not seen in deer, indicating that the rate of infection by HEV under natural conditions is extremely low in deer, and suggesting that deer do not play an important role as a reservoir of HEV in Japan.

This study included 254 serum samples from deer captured in Hyogo, where the first deer positive for HEV RNA was found [24]. The prevalence of the antibody-positive rate was 3.1% (5/132) in female and 2.5% (3/122) in male deer, and the difference

between the sexes was not statistically significant ($P>0.05$). These antibody-positive rates are also not significantly different from those in other areas, including Hokkaido, Iwate, and Miyazaki prefecture. The age of anti-HEV IgG-positive deer was 0–8 years, and no significant correlation between age and prevalence was observed. We also tested HEV RNA in 36 serum samples from deer captured in the same area in Hyogo prefecture where the deer that was positive for HEV RNA was reported. However, we were not able to amplify any HEV sequences in these samples.

Since wild boars are prevalent throughout Japan, with the exception of Hokkaido, and they seem to be eventually infected with HEV, the virus is spread throughout their habitat via their stools. Because wild deer and wild boars share this environment, wild deer might be exposed to HEV. Only low-titer anti-HEV IgG was detected in deer serum in this study, suggesting that either the antibody detected in this study was not induced by HEV infection or that deer have low sensitivity to HEV. If deer were to occasionally come into contact with a small amount of HEV, but were not susceptible to HEV, then a strong immune response to HEV might not be induced.

In summary, the prevalence of anti-HEV IgG in sika deer was lower than the prevalence in two possible reservoirs, pigs and wild boars, and no HEV RNA was detected in 254 sera, 88 stool and 159 liver tissue samples, indicating that wild deer may not be a reservoir of HEV in Japan.

Acknowledgments

The authors thank Tomoko Mizoguchi for her secretarial work. This study was supported in part by grants-in-aid for Research on Emerging and Re-emerging Infectious Diseases, Research on Hepatitis, and Research on Food Safety from the Ministry of Health, Labor and Welfare, Japan.

We also would like to thank the Hyogo Hunting Association, the Okoppe, Shari, and Kushiro branches of the Hokkaido Hunting Association, the Shiretoko Nature Center, the Deer Research group in Lake Toya, and Envision for their extensive help in collecting the samples. This work was supported by a grant from JSPS (no. KAKENHI16380202) and by a grant from the 21st Century COE Program, "Program of Excellence for Zoonosis Control," from the Ministry of Education, Science, Sports and Culture of Japan.

References

1. Balayan MS (1997) Epidemiology of hepatitis E virus infection. J Viral Hepat 4: 155–165
2. Buisson Y, Grandadam M, Nicand E, Cheval P, van Cuyck-Gandre H, Innis B, Rehel P, Coursaget P, Teyssou R, Tsarev S (2000) Identification of a novel hepatitis E virus in Nigeria. J Gen Virol 81: 903–909
3. Emerson SU AD, Arankalle A, Meng XJ, Purdy M, Schlauder GG, Tsarev SA (2005) Hepevirus. In: Fauquet CM, Maniloff J, Desselberger U, Ball LA (eds) Virus taxonomy, VIIIth Report of the ICTV. Elsevier/Academic Press, London, pp 853–857
4. Hijikata M, Hayashi S, Trinh NT, Ha le D, Ohara H, Shimizu YK, Keicho N, Yoshikura H (2002) Genotyping of hepatitis E virus from Vietnam. Intervirology 45: 101–104
5. Jameel S (1999) Molecular biology and pathogenesis of hepatitis E virus. Expert Rev Mol Med 1999: 1–16
6. Koike H, Ohtaishi N (1985) Prehistoric hunting pressure estimated by the age composition of excavated sika deer (*Cervus nippon*) using the annual layer of tooth cement. J Archaeol Sci 12: 443–456
7. Li TC, Yamakawa Y, Suzuki K, Tatsumi M, Razak MA, Uchida T, Takeda N, Miyamura T (1997) Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol 71: 7207–7213
8. Li TC, Zhang J, Shinzawa H, Ishibashi M, Sata M, Mast EE, Kim K, Miyamura T, Takeda N (2000) Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. J Med Virol 62: 327–333
9. Li TC, Chijiwa K, Sera N, Ishibashi T, Etoh Y, Shinohara Y, Kurata Y, Ishida M, Sakamoto S, Takeda N, Miyamura T (2005) Hepatitis E virus transmission from wild boar meat. Emerg Infect Dis 11: 1958–1960
10. Li TC, Saito M, Ogura G, Ishibashi O, Miyamura T, Takeda N (2006) Serologic evidence for hepatitis E virus infection in mongoose. Am J Trop Med Hyg 74: 932–936
11. Magden J, Takeda N, Li T, Auvinen P, Ahola T, Miyamura T, Merits A, Kaariainen L (2001) Virus-specific mRNA capping enzyme encoded by hepatitis E virus. J Virol 75: 6249–6255
12. Maila HT, Bowyer SM, Swanepoel R (2004) Identification of a new strain of hepatitis E virus from an outbreak in Namibia in 1995. J Gen Virol 85: 89–95
13. Meng XJ, Purcell RH, Halbur PG, Lehman JR, Webb DM, Tsareva TS, Haynes JS, Thacker BJ, Emerson SU (1997) A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci USA 94: 9860–9865
14. Meng XJ, Halbur PG, Shapiro MS, Govindarajan S, Bruna JD, Mushahwar IK, Purcell RH, Emerson SU (1998) Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J Virol 72: 9714–9721

15. Meng XJ (2003) Swine hepatitis E virus: cross-species infection and risk in xenotransplantation. Curr Top Microbiol Immunol 278: 185–216
16. Purcell RH, Emerson SU (2001) Hepatitis E virus. In: Knipe DM, Howley PM (eds) Fields virology, vol 1. Lippincott Williams & Wilkins, Philadelphia, pp 3051–3061
17. Schlauder GG, Dawson GJ, Erker JC, Kwo PY, Knigge MF, Smalley DL, Rosenblatt JE, Desai SM, Mushahwar IK (1998) The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. J Gen Virol 79 (Pt 3): 447–456
18. Schlauder GG, Mushahwar IK (2001) Genetic heterogeneity of hepatitis E virus. J Med Virol 65: 282–292
19. Takahashi K, Iwata K, Watanabe N, Hatahara T, Ohta Y, Baba K, Mishiro S (2001) Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology 287: 9–12
20. Takahashi K, Kang JH, Ohnishi S, Hino K, Miyakawa H, Miyakawa Y, Maekubo H, Mishiro S (2003) Full-length sequences of six hepatitis E virus isolates of genotypes III and IV from patients with sporadic acute or fulminant hepatitis in Japan. Intervirology 46: 308–318
21. Takahashi M, Nishizawa T, Miyajima H, Gotanda Y, Iita T, Tsuda F, Okamoto H (2003) Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J Gen Virol 84: 851–862
22. Takahashi M, Nishizawa T, Okamoto H (2003) Identification of a genotype III swine hepatitis E virus that was isolated from a Japanese pig born in 1990 and that is most closely related to Japanese isolates of human hepatitis E virus. J Clin Microbiol 41: 1342–1343
23. Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, Reyes GR (1991) Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 185: 120–131
24. Tei S, Kitajima N, Takahashi K, Mishiro S (2003) Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 362: 371–373
25. Tei S, Kitajima N, Ohara S, Inoue Y, Miki M, Yamatani T, Yamabe H, Mishiro S, Kinoshita Y (2004) Consumption of uncooked deer meat as a risk factor for hepatitis E virus infection: an age- and sex-matched case-control study. J Med Virol 74: 67–70
26. Velazquez O, Stetler HC, Avila C, Ornelas G, Alvarez C, Hadler SC, Bradley DW, Sepulveda J (1990) Epidemic transmission of enterically transmitted non-A, non-B hepatitis in Mexico, 1986–1987. JAMA 263: 3281–3285
27. Wang Y, Ling R, Erker JC, Zhang H, Li H, Desai S, Mushahwar IK, Harrison TJ (1999) A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. J Gen Virol 80 (Pt 1): 169–177
28. Wang Y, Zhang H, Ling R, Li H, Harrison TJ (2000) The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. J Gen Virol 81: 1675–1686
29. Wu JC, Chen CM, Chiang TY, Tsai WH, Jeng WJ, Sheen IJ, Lin CC, Meng XJ (2002) Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan. J Med Virol 66: 488–492
30. Yazaki Y, Mizuo H, Takahashi M, Nishizawa T, Sasaki N, Gotanda Y, Okamoto H (2003) Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be foodborne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol 84: 2351–2357