HFRS-related viruses, except for the rhesus monkey and Syrian golden hamster models[4,12]. Although hantavirus pathogenesis in the mouse model differs from that in humans, we found that all of the hantaviruses that are associated with severe HFRS, such as AMR, FE, and HTN viruses, are potentially virulent for newborn mice. To date, there is no adult mouse model of lethality due to HFRS-related hantaviruses. Since the adult mouse model is extremely useful for vaccine evaluation, virulence and pathological analyses in adult mice are underway using the AMR genotype virus in adult mice.

Acknowledgments

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Genetic and antigenic characterization of the Amur virus associated with hemorrhagic fever with renal syndrome

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Abstract

The genetic and antigenic characteristics of the Amur (AMR) and Far East (FE) virus lineages, which are both within the genus *Hantavirus*, were studied. Representative viruses, H5 and B78 for AMR and Bao 14 for FE, were used. The entire small (S) and medium (M) segments, except for the 3′- and 5′-ends, were sequenced. The deduced amino acid sequences of AMR had 96.7 and 92.0–92.2% identities with the Hantaan (HTN) virus in the S and M segments, respectively. The amino acid sequences of FE had 99.1 and 97.9% identities in the S and M segments, respectively. The three viral strains and HTN virus had similar binding patterns to a panel of monoclonal antibodies (MAbs), except that one MAb did not bind AMR. However, sera from *Apodemus peninsulae*, naturally infected with AMR virus, neutralized homologous viruses at 1:160 to 1:320 dilutions and HTN at 1:20 to 1:40 dilutions. The anti-AMR serum neutralized homologous viruses at a 1:80 dilution and HTN at a 1:40 dilution. The anti-HTN serum did not neutralize AMR (<1:40 dilution), although it had a high neutralizing titer (1:320) against the homologous virus. Therefore, we suggest that AMR virus may constitute a distinct serotype within the genus *Hantavirus*.

Keywords: Bunyaviridae; Hantavirus; Hantavirus infections; Hemorrhagic fever with renal syndrome; Hantaan; Amur

Hantavirus, which is a member of the genus *Hantavirus* of the family Bunyaviridae, is enveloped, and contains tripartite, single-stranded, negative-sense RNA segments. The small (S), medium (M), and large (L), RNA segments encode a nucleocapsid protein (NP), surface glycoproteins G1 and G2, and the viral polymerase, respectively (Antic et al., 1991; Schmaljohn and Dalrymple, 1983; Hung et al., 1983; Plyusnin et al., 1996). It has been shown that the hantavirus NP acts as the major cross-reactive antigen between the different antigenic groups of hantaviruses (Sheshberadaran et al., 1988). The hantavirus G1 and G2 proteins mediate many important biological properties, such as virulence, neutralization, hemagglutination, and cell fusion (Elliot, 1990). Studies on the antigenic characterization of the Hantaan (HTN) virus envelope glycoproteins have shown that the G1 and G2 proteins contain major antigenic determinants that play an important roles in the induction of protective humoral immunity to hantavirus infection (Arikawa et al., 1989; Dantas et al., 1986; Lundkvist and Niklasson, 1992; Ruo et al., 1991). The deduced amino acid sequences of certain hantaviruses have shown that the L proteins are more conserved than the gene products of the M or S segment (Antic et al., 1991; Schmaljohn, 1990; Stohwasser et al., 1991).

Some hantaviruses cause severe human illnesses, such as the hemorrhagic fever with renal syndrome (HFRS) that is endemic to Eurasia, and the hantavirus pulmonary syndrome (HPS) that is endemic to the Americas (CDC, 1993; Clement et al., 1997; Hughes et al., 1993). It is well known that hantaviruses are transmitted to humans via infectious aerosols from persistently infected rodents (Tsai, 1987) or via contaminated saliva in animal bites (Douron et al., 1984). Thus, both HFRS and HPS are considered important rodent-borne zoonoses (Lee and Van der Groen, 1989; Nichol et al., 1993; Zaki et al., 1995).

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The HTN and Seoul (SEO) viruses cause HFRS in Asia (Lee et al., 1978; Kitamura et al., 1983), and represent a considerable public health problem in China with almost 100,000 cases reported annually (Lee, 1996; Song, 1999). Recent studies have revealed that the HTN and SEO viruses in China are carried by *Apodemus agrarius* and *Rattus norvegicus*, respectively, and these viruses are the major causes of HFRS (Song et al., 1984; Kariwa et al., 2001). Many hantaviruses have been isolated from various rodent species and patients in China. These isolates were found to be antigenically related to HTN or SEO (Tang et al., 1991; Liang et al., 1994). Furthermore, a distinct novel hantavirus type, NC167, has been isolated recently from *Niviventer confucianus*. Nucleotide and amino acid comparisons confirm that NC167 represents a novel hantavirus type, which have been named provisional "Da Bie Shan virus". In addition, a new subtype of SEO virus, Gou3, has been isolated from *Rattus rattus* (Wang et al., 2000).

Far East Russia is an endemic area for the clinically severe form of HFRS, and many different hantaviruses have been found in various rodent species and in patients in this area (Kariwa et al., 1999). Recently, two hantavirus lineages, which have been designated Amur (AMR) and Far East (FE), were identified in severe HFRS patients in the same area (Yashina et al., 2000; Miyamoto et al., 2003). It has been revealed that *A. peninsula* is the reservoir animal for the AMR lineage (Yashina et al., 2001; Lokugamage et al., 2002). These lineage viruses appear to be related to HTN viruses but show divergence, according to limited sequence analyses. Although the AMR and FE lineages cause severe HFRS in humans, the genetic and antigenic properties of these lineages have not been characterized adequately. Our previous findings suggest a variety of lineages among the HTN-related viruses in China, and indicate that viruses that are closely related to AMR and FE also exist in China (Wang et al., 2000; Lokugamage et al., 2002). Phylogenetic analysis, based on partial M gene nucleotide sequences, suggests that two Chinese human isolates, H5 and B78, belong to the AMR lineage, while another Chinese isolate from *A. agrarius*, Bao 14, belongs to the FE lineage. Therefore, we used these isolates to elucidate the genetic and antigenic properties of AMR and FE lineages, which are related to, but show genetic divergence from, the HTN virus.

The hantaviruses used in this study were propagated in Vero E6 cells that were cultivated in Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 5% fetal calf serum, kanamycin and L-glutamine. All viruses used in this study were handled in a biosafety level 3 laboratory. The Hantaan 76-118 and SR-11 strains were used as prototypes of the HTN and SEO viruses, respectively. The three Chinese isolates (H5, B78, and Bao14) examined in this study were kindly provided by Dr. C. Hung of the Chinese Academy of Preventive Medicine. H5 and B78 were isolated from HFRS patients, while Bao 14 was isolated from *A. agrarius*.

Five-week-old specific-pathogen-free male ICR mice (SLC, Hamamatsu, Japan) were inoculated subcutaneously (sc) with 1×10^2–1.6×10^3 FFU of HTN 76-118, SR-11, H5, B78, and Bao14, and were supplied with food and water ad libitum. The mice were bled for immune sera 2 months later by cardiac puncture under anesthesia. All of the animal experiments were carried out in biosafety level 3 facilities according to the guidelines for animal experimentation of the Graduate School of Veterinary Medicine, Hokkaido University.

The Vero E6 cells were infected with viruses and spotted onto 24-well slides. After incubation at 37 °C for 4 h, the slides were fixed with cold acetone. The air-dried slides were used as antigen slides. A panel of monoclonal antibodies (MAbs) that recognize glycoproteins G1 and G2, and the nucleocapsid protein of HTN or SEO virus was used in a standard IFA on acetone-fixed, H5, B78, Bao 14, HTN 76-118, and SR-11 infected Vero E6 cells (Arikawa et al., 1989; Yoshimatsu et al., 1996). Briefly, serially diluted Mabs (1:1 to 1:1000 for MAbs that were derived from culture supernatant and 1:100 to 1:100,000 for Mabs that were derived from ascitic fluids) were spotted onto infected Vero E6 cells and incubated for 1 h at 37 °C. After three washes with phosphate-buffered saline (PBS), fluorescein isothiocynate (FITC)-conjugated antibody to mouse IgG was added to the cells (ICN pharmaceuticals Inc., Aurora, OH). Incubation at 37 °C for 1 h was followed by three washes with PBS. Specific antibody binding was detected by fluorescent microscope.

The endpoint titers of the neutralizing antibodies were determined by FRNT. Mouse immune sera, which were prepared against H5, B78, Bao14, HTN 76-118, and SR-11 viruses, were used to analyze the serological relationships among AMR and FE genotypes and the HTN virus. Serially diluted antisera (100 μl) were mixed with equal volume of the stock viruses (100 focus-forming units/100 μl). After 1 h incubation at 37 °C the mixtures (100 μl/well) were inoculated onto Vero E6 cells monolayers that were grown in eight-well slides. After adsorption for 1 h at 37 °C, the inocula were removed and MEM that contained 1.5% carboxymethyl cellulose (CMC) was layered onto the cells. The slides were incubated in a CO_2 incubator at 37 °C for 5–7 days. After this incubation period, the monolayers were washed with PBS, fixed with cold acetone, and air-dried. Mouse immune sera to the HTN virus were added to the fixed Vero E6 cells. Incubation for 1 h at 37 °C was followed by three washes with PBS. The FITC-conjugated antibody to mouse IgG was added to the cells and incubated at 37 °C for 1 h. FITC-stained foci were counted under a fluorescence microscope. The FRNT titer was determined as the highest dilution of the serum that showed 80% or higher reduction of focus formation.

Total cellular RNA was extracted from the virus-infected Vero E6 cells using Isogen (Nippon Gene Co. Ltd., Osaka, Japan) according to the manufacturer's instructions. Reverse transcription was carried out at 42 °C for 30 min with

superscript II and Random primers (Gibco-BRL, Rockville, MD). The amplification of the S and M segments was similar to the method described previously (Lokugamage et al., 2002). Totally 32 primers were used for amplification or sequencing the S and M segments. The amplified segments were sequenced directly using a Big Dye terminator (Applied Biosystems, Lincoln Centre Drive, Foster City, CA, USA) and an ABI 310 genetic analyzer.

The CLUSTALX program package (Version 1.81;URL: ftp://ftp-igbmc.u-strasbg.fr/pub/clustalx) was used to generate the phylogenetic tree using the neighbor-joining method with 1000 bootstrap replicates. The hantavirus sequences used in the comparisons were obtained from GenBank.

In our previous study, limited sequencing analysis suggested the existence in China of viruses that are closely related to the AMR and FE lineages (Lokugamage et al., 2002). Although the sequences of the AMR and FE lineages have been identified in HFRS patients, and these lineages may be the major cause of the disease in Far East Russia, the characterization of these viruses has not been defined precisely and has been limited to the analysis of partial gene sequences. Therefore, the information needed to classify these viruses has been lacking. In addition, these viruses appear to exist in a vast area of the Eurasian continent, including Far East Russia, China, and Korea, and they cause a number of HFRS cases. Therefore, we sequenced the G2 regions on the M segments of these three Chinese isolates and performed phylogenetic analyses (Fig. 1). The phylogenetic tree clearly shows that H5 and B78 belong to the AMR lineage, which includes sequences derived from HFRS patients and

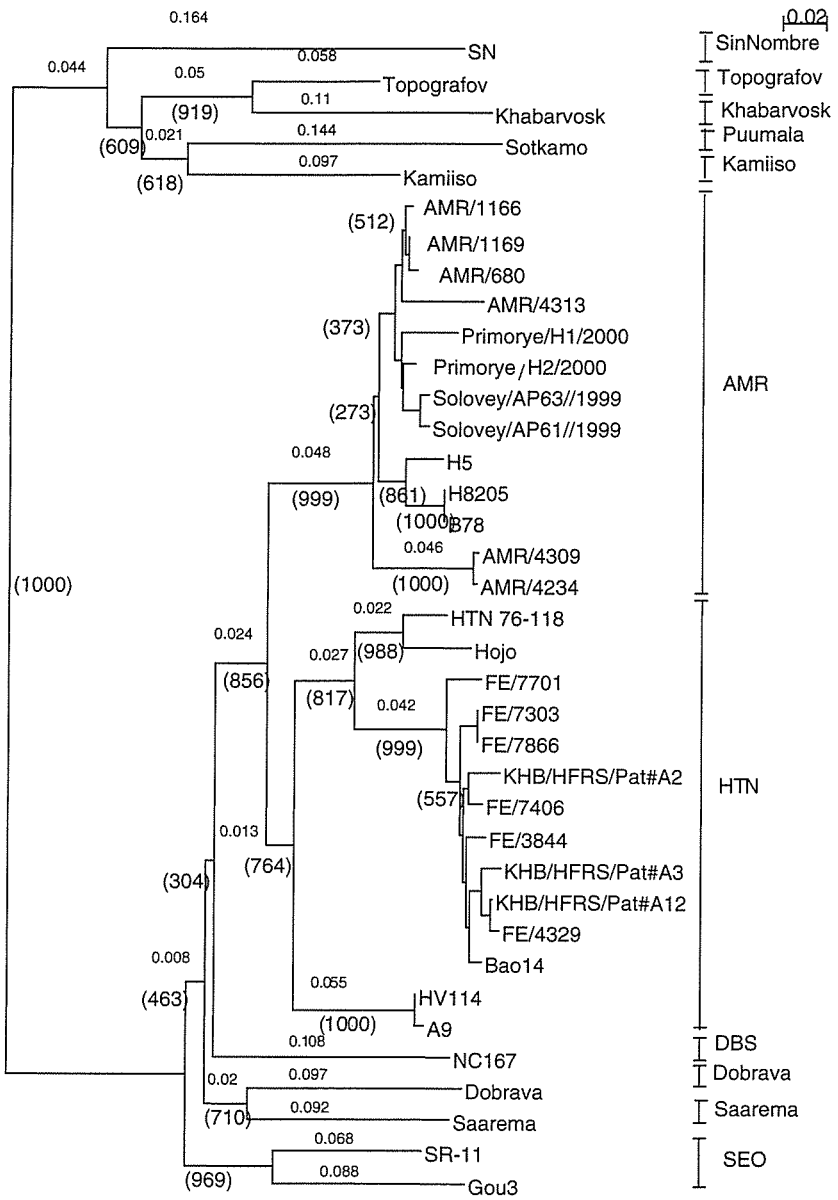

Fig. 1. Phylogenetic tree of the hantavirus partial M (nt 2736–2968) segments. The tree was constructed using the CLUTALX (Version 1.81) program. The numbers above the branches are the distances and those in parentheses are the bootstrap support values for 1000 replicates.

A. peninsulae. In contrast, Bao 14 belongs to the FE lineage, which includes sequences from HFRS patients.

To reveal detailed genetic information on the AMR and FE lineages, we amplified and sequenced the entire S segments of the H5, B78, and Bao 14 strains, except for the 20 nucleotides at the 3′- and 5′-ends (AB127996, AB127997, and AB127998). The nucleotide and amino acid identities among the AMR lineage (H5, B78, and SL/AP/63) were 90.9–99.8 and 98.6–100%, respectively. The AMR lineage and Bao 14 had 85.2–86.0 and 97.2–97.7% identities at the nucleotide and amino acid levels, respectively. The S gene of AMR lineage had diversities, at 16–17 and 3–4% in nucleotide and amino acid levels, respectively, with the prototype HTN virus. Meanwhile, the S gene of FE lineage differed by only 10 and 0.9% difference in nucleotide and amino acid levels, respectively, with the prototype HTN virus.

To provide further insight into the genetic relationship between the AMR- and FE-lineage viruses, the entire M segments of H5, B78, and Bao 14 (AB127993, AB127994, and AB127995) were sequenced and compared. The nucleotide and amino acid identities among the AMR lineage (H5, B78, and H8205) were 96.3–99.5 and 98.5–99.2%, respectively. The nucleotide and amino acid identities between AMR and the other HTN-related viruses (A9, Bao14, HTN 76-118, Lee, and Hojo) were approximately 80 and 91–92%, respectively. Bao 14 had 87.8 and 97.9% identities

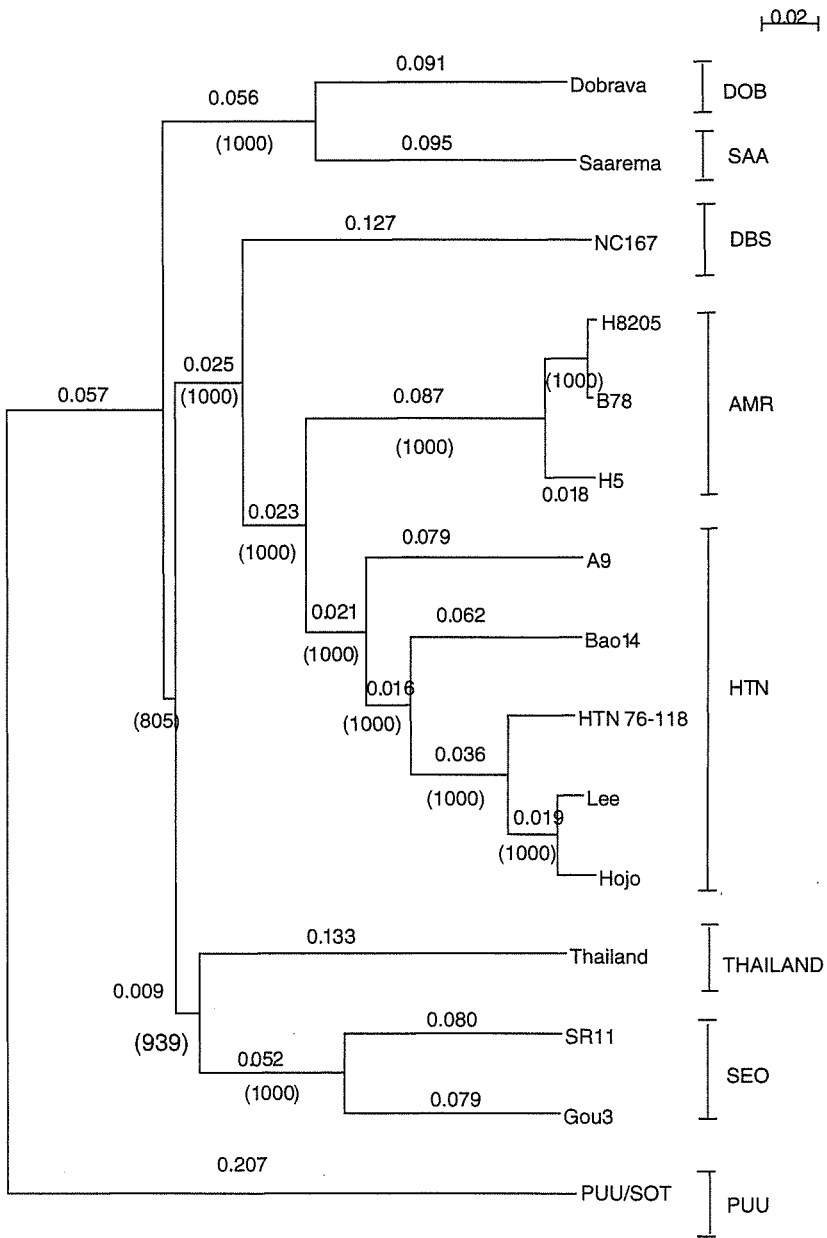

Fig. 2. Phylogenetic tree of the hantavirus entire M segments except for 20 nucleotides at both ends. The tree was constructed using the CLUTALX (Version 1.81) program. The numbers above the branches are the distances and those in parentheses are the bootstrap support values for 1000 replicates.

with HTN 76-118 at the nucleotide and amino acid levels, respectively.

The phylogenetic tree on the complete M segments showed that the H5 and B78 viruses formed a single cluster, together with H8205, which is one of AMR viruses, with high bootstrap support values. It was also clear that Bao 14 virus was monophyletic with the HTN virus (Fig. 2). These results indicate the AMR lineage viruses may form a distinct branch from the HTN clade but the FE may be the subtype of the HTN virus.

Ten out of fourteen amino acid mutations in the S segments of the aligned sequences were unique to the AMR-lineage viruses (data not shown). With respect to the amino acid alignment of the glycoprotein precursor (GPC), six possible asparagine-linked glycosylation sites on GPC were totally conserved in the AMR, FE, and HTN viruses (Fig. 3). In our previous study, we identified signature amino acids for AMR lineage that was based on the deduced partial amino acid sequences of GPC. The present data show that these signature amino acids are present in deduced

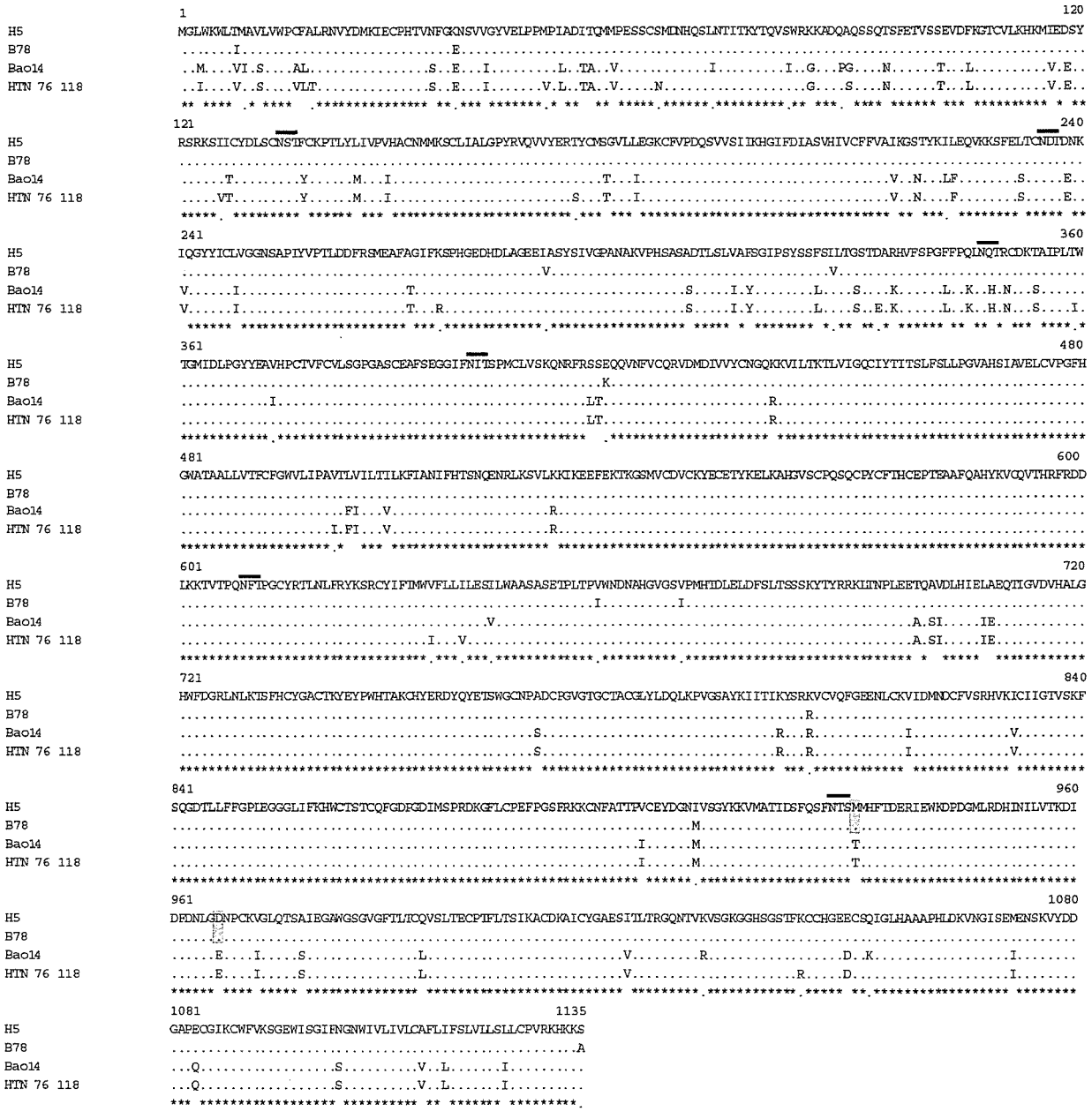

Fig. 3. Multiple alignment of the deduced amino acid sequences of the G1 and G2 regions of hantaviruses. The amino acid sequences were analyzed using the CLUTALX (Version 1.8) program. The amino acid positions indicated above the sequences are based on those of HTN 76-118. The first line shows the deduced amino acid of H5. The dots represent amino acids that are identical to those at corresponding positions in the H5 sequence. Amino acids that differ from those in the H5 sequence are indicated at the relevant positions. The signature amino acids for AMR sequences, which were identified in our previous study, are shaded. Possible asparagine-linked glycoprotein sites are indicated by lines over the corresponding sites.

Table 1
Antigenic characterization of HTN 76-118, Bao 14, H5, B78, and SR-11 by a panel of monoclonal antibodies

Virus	Antigenic site	Mab	HTN 76-118	Bao14	H5	B78	SR 11
HTN	G1-a	6D4	+	+	+	+	−
		10 F 11	+	++	+	+	−
	G1-b	16D2/3D5	+	+	+	+	−
		2D5	+	+	−	−	−
	G2-a	HCO2	++	+	++	++	+
		16E 6	++	+	+	+	−
	G2-b	EBO6	+	+	+	+	+
	G2-c	11 E 10 2-2	++	++	++	++	−
	G2-d	17G6	+	+	+	+	+
		3D7	++	++	+	+	+
		5 B 7	+	+	++	+	+
	G2-e	20 D 3	+	++	+	+	+
	G2-f	8 E 10/ 23G	+	+	+	+	+
		10-1/18 F					
		5/1 C 6					
		7G6/1 G 6	+	++	++	+	+
		3 B 6	+	+	+	+	++
		1G8	++	++	++	+	+
	NP-I	ECO2	++	++	++	++	+
	NP-III	C16D11	++	++	++	++	+
		C24B4	++	++	++	++	−
		F23A1	++	++	+	+	+
SEO	NP	2 E 8	−	−	−	−	++

Antibody reactivity is defined as: −, <10; +, 10–100; ++, 1000.

amino acid sequences of H5 and B78, which confirm that these two strains belong to the AMR lineage.

The antigenic characterization of the AMR and FE lineages was performed with a panel of MAb to glycoproteins G1 or G2 and NP (Table 1). Almost all MAb used in this study were reacted with the HTN, AMR, and FE viruses with more or less in similar patterns, except for the 2D5 MAb, which recognizes G1-b site. This particular MAb did not react with the H5 and B78 viruses, which implies that these viruses contain amino acid changes in the epitope for this MAb.

However, cross-neutralization analysis showed that the AMR lineage viruses had apparently distinct antigenicities as compared to that of the prototype HTN virus (Table 2). The titers of the anti-H5 and anti-B78 sera to homologous viruses (1:80) were two-fold higher than that against the HTN virus (1:40), while the titer of anti-HTN immune serum to the homologous HTN virus (1:320) was eight-fold higher than that against the H5 and B78 viruses (<1:40). In addition, when we used sera from two *A. peninsulae* which were captured in the suburb of Vladivostock and which had AMR sequences (SL/AP61/2000 and SL/AP63/2000) in their lungs (Lokugamage et al., 2002), the titers of those two sera to the H5 and B78 viruses (1:160 to 1:320) were eight-fold higher than those against the HTN virus (1:20 to 1:40). Furthermore, the titer of anti-Bao 14 to the homologous virus (1:1280) was 16–32-fold higher than those against the AMR lineage viruses (1:40 to 1:80) but was

Table 2
Antigenic characterization by cross focus reduction neutralization test

Antisera	Species	Neutralization titer to the following viruses[a] H5	B78	Bao14	HTN 76-118	SR-11
H5	Mouse	80[b]	80	80	40	<40
B78	Mouse	80	80	160	40	<40
#61[c]	*A. peninsulae*	160	320	20	20	ND[d]
#63[c]	*A. peninsulae*	320	320	40	40	ND
Bao14	Mouse	40	80	1280	320	40
HTN 76–118	Mouse	<40	<40	320	320	<40
SR-11	Rabbit	10	<10	<10	10	160

[a] Neutralization titer was expressed as a reciprocal of the highest dilution which showed 80% or more of inhibition of the virus focus formation.
[b] Neutralization titer with homologous immune sera was underlined.
[c] Sera from *Apodemus peninsulae* which were captured in the suburb of Vladivostock and had Amur sequences in their lungs (34).
[d] Not done.

four-fold higher than that against the HTN virus. These results indicate that the H5 and B78 viruses are antigenically distinguishable from HTN virus with a four-fold titer difference in two-way cross-neutralization. Further, the anti-HTN immune serum titer to the homologous HTN virus (1:320) was similar to that of Bao 14 virus (1:320) (Table 2).

The three-dimensional neutralizing epitope in AMR lineage viruses may differ from that in the HTN virus. This finding is important, as the hantavirus vaccine strains that have been developed in China and Korea are closely related to the prototype of HTN virus. Thus, these vaccines may not protect against infection with AMR lineage viruses.

Hantavirus classification is very complicated because of the geographic variation among hantaviruses that are carried by single or several closely related host species. Elliot et al. (1999) suggested the following criteria for the distinction of viruses in the genus *Hantavirus*: (a) a unique ecological niche for each virus species, i.e., a clear association of a new hantavirus with a different primary rodent reservoir species or subspecies; (b) at least 7% amino acid sequence difference from previously characterized hantaviruses (on comparison of the complete glycoprotein precursor and nucleocapsid protein sequences); (c) at least a four-fold difference in two-way cross-neutralization tests; (d) absence of genetic reassortment in nature.

Accordingly, the AMR lineage viruses are carried by *A. peninsulae* not by *A. agrarius* (Lokugamage et al., 2002). Furthermore, the amino acid diversity of the glycoprotein precursor is more than 7% as compared to other HTN viruses. In addition, the difference in neutralizing titer is greater than eight-folds in two-way cross-neutralization tests. There is no information about re-assortmants between the AMR and other hantaviruses. Therefore, AMR meets most of the criteria for a distinct virus. In contrast, the FE viruses do not meet these criteria.

Therefore, we suggest that AMR virus may constitute a distinct virus in the genus *Hantavirus*, and that FE may be a subtype of the prototype HTN virus.

Since there are many other lineages that are equivalent to AMR, which are antigenically closely related with the HTN clade (Wang et al., 2000), we should reconsider the classification of those viruses, which have traditionally been classified as HTN viruses.

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Epizootiological and Epidemiological Study of Hantavirus Infection in Japan

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Abstract: **Epizootiological surveys on hantavirus infections in rodents were carried out in various areas of Japan, including the four major islands of Hokkaido, Honshu, Shikoku, and Kyushu from 2000 to 2003. A total of 1,221 rodents and insectivores were captured. Seropositive animals were found in *Apodemus* (*A.*) *speciosus* (5/482, 1.0%), *Rattus* (*R.*) *norvegicus* (4/364, 1.1%), *R. rattus* (3/45, 6.7%), and *Clethrionomys* (*C.*) *rufocanus* (7/197, 3.6%). The partial S segment was amplified from one seropositive *R. rattus* captured at Hakodate. The nucleotide sequence showed 96% identity with the Seoul virus (SEOV) prototype strain SR-11. In addition, we conducted an epidemiological survey on human hantavirus infection in a high-risk population, the personnel of the Japan Ground Self-defense Force on Hokkaido. One out of 207 human blood samples was positive for anti-hantavirus antibody by IFA, ELISA, and WB analysis. The result of the serotype specific ELISA indicates that this individual acquired SEOV infection. This study indicates that *A. speciosus*, *R. norvegicus*, *R. rattus*, and *C. rufocanus* carry hantaviruses as the reservoir animals in Japan. Infected *R. rattus* and *R. norvegicus* in port areas could be the sources of human SEOV infection and a threat to travelers and individuals working in seaports.**

Key words: **Hantavirus, Rodent, Epidemiology, Epizootiology**

Hantaviruses are causative agents of two human illnesses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). The animal reservoirs of hantaviruses are various rodent species, which, when infected, do not show any symptoms and carry the virus for long periods. Humans acquire hantavirus infection by inhalation of virus-containing excreta from infected animals. More than 20 serotypes or genotypes of hantaviruses have been reported, and each virus has a specific rodent reservoir. Because the phylogenies of the viruses and the reservoir rodents are topologically identical, it is generally believed that hantaviruses and rodents have co-evolved (17). The factors responsible for the emergence of human hantavirus infections include changes in ecological factors and changes in human activities. The most important risk factor is close contact with rodents as a result of agricultural, forestry, or military activities (14).

HFRS is caused by Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), and Dobrava virus (DOBV) in Eurasia; these viruses are carried by

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Abbreviations: AMRV, Amur virus; ANDV, Andes virus; BAYV, Bayou virus; BCCV, Black Creek Canal virus; CMC, carboxymethyl cellulose; DOBV, Dobrava virus; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FFU, focus forming unit; FITC, fluorescein isothiocyanate; FRNT, focus reduction neutralization test; HFRS, hemorrhagic fever with renal syndrome; HPS, hantavirus pulmonary syndrome; HTNV, Hantaan virus; IFA, indirect immunofluorescent-antibody assay; MEM, minimum essential medium; NP, nucleocapsid protein; NYV, New York virus; OD, optical density; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PUUV, Puumala virus; PVDF, polyvinylidene fluoride; SAAV, Saaremaa virus; SEOV, Seoul virus; SNV, Sin Nombre virus; WB, Western blot.

Apodemus (*A.*) *agrarius*, *Rattus* (*R.*) *norvegicus* and *R. rattus*, *Clethrionomys* (*C.*) *glareolus*, and *A. flavicollis*, respectively (3). Recently, Saaremaa virus (SAAV) was identified as the causative agent of a mild form of HFRS in Europe, and *A. agrarius* was found to be the carrier (15, 18). There is serological evidence of human SAAV infections in Estonia (4), and SAAV might have been the cause of an HFRS outbreak in Russia in the 1990 s (18).

On the American continent, HPS is caused by Sin Nombre virus (SNV), New York virus (NYV), Black Creek Canal virus (BCCV), Bayou virus (BAYV), and Andes virus (ANDV), which are carried by *Peromyscus* (*P.*) *maniculatus*, *P. leucopus*, *Sigmodon hispidus*, *Oryzomys palustris*, and *Oligoryzomys longicaudatus*, respectively (16, 17).

About 200,000 HFRS cases are reported annually throughout the world (10). A wide variety of hantaviruses responsible for HFRS have been found in East Asia (19). About 50,000 to 100,000 HFRS cases are reported annually in China, where HTNV and SEOV are responsible for most of the cases (20). In addition, Far East Russia is well known as an endemic area for HFRS. About 100 to 200 HFRS patients are reported annually in that region. Recently, a distinct type of hantavirus, Amur virus (AMRV), was identified in HFRS patients in Far East Russia (23). Our previous studies revealed that *A. peninsulae* is the reservoir animal for AMRV, as well as revealing antigenic and genetic evidence of a distinct hantavirus serotype (12, 13). Furthermore, Khabarovsk and Vladivostock viruses were also identified in the same region (5, 6).

In Japan, there have been two outbreaks of HFRS since the 1960s. One was reported in the Umeda district in Osaka city in the 1960s (22). The source of the infection is believed to have been urban rats (*R. norvegicus*). The other outbreak was reported in various animal facilities in the country between 1970 and 1984, and the human infections were related to contact with laboratory rats (*R. norvegicus*) (9, 11). Since 1985 to the present, there have been no reported HFRS cases. However, seropositive *R. norvegicus* have been identified in ports and reclaimed areas in different locations throughout the country (2). In addition, Puumala-related viruses are widely distributed in *C. rufocanus* on Hokkaido, the northern-most major island of Japan (6, 8).

Although our recent study identified anti-hantavirus antibodies among patients with hepatitis of unknown etiology in Japan, the prevalence of the antibody is very low in the general population (7). It is unclear why Japan has few HFRS patients in spite of the highly endemic nature of the disease in surrounding countries. To clarify this question, a large-scale epizootiological study targeting indigenous rodents was essential. Therefore, we carried out epizootiological surveys in rodents from various areas of Japan, including the four major islands of Hokkaido, Honshu, Shikoku, and Kyushu, to determine the endemic areas and the reservoir animals.

Materials and Methods

Cells and viruses. Hantavirus strains HTN 76-118, SR-11, and Sotkamo, were propagated as representative strains of HTNV, SEOV, and PUUV, respectively, in Vero E6 cells grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FCS). The cultured medium of the infected cells was harvested 7–14 days later and stored as stock virus at −80 C. All experiments with live viruses were carried out in a P3 containment room.

Rodent sera. A total of 1,221 rodent and insectivore sera were collected from field surveys on Hokkaido, Honshu, Shikoku, Kyushu, and Tsushima Island in Japan from 2000 to 2003. The species captured were *C. rufocanus*, *C. rutilus*, *A. speciosus*, *A. argentius*, *R. norvegicus*, *R. rattus*, *M. montebelli*, *E. smithi*, *M. minutus*, *M. musculus*, *C. dsinezumi*, and *U. talpoides*. Blood samples were collected from live animals by cardiac puncture under anesthesia. Blood samples from dead animals were collected on filter papers (Toyo, Tokyo). After sampling, the papers with absorbed blood were air-dried, cut into four pieces, and collected into microcentrifuge tubes containing 0.4 ml of phosphate buffered saline (PBS). The tubes were held at 4 C over night and then heated at 56 C for 30 min. The tubes were centrifuged at 10,000 revolutions per minitue for 10 min and the supernatants were transferred to new tubes as 1:10 diluted sera. All collected sera were stored at −40 C until use.

Human sera. A total of 207 human sera were collected from the personnel of the Japan Ground Self-defense Force, who had been training on Hokkaido for several years. The blood samples were processed as described above for the rodent samples and stored at −40 C until use.

Indirect immunofluorescent antibody assay (IFA). Vero E6 cells in 75-cm^2 flasks were infected with HTNV, SEOV, or PUUV and cultured for 6, 7, or 11 days, respectively. The cells were collected by trypsinization and seeded onto 24-well slides. The slides were incubated for 4 hr at 37 C in a CO_2 incubator. The cells were fixed with cold acetone for 20 min and air dried for 1 hr. The prepared slides were stored at −40 C until use. The sera were spotted onto the 24-

well slides and incubated for 1 hr at 37 C. The slides were washed with PBS and spotted with fluorescein isothiocyanate (FITC)-conjugated protein G (Zymed, Laboratories, Inc., San Francisco, Calif., U.S.A.). After incubation at 37 C for 1 hr, the slides were washed and examined under fluorescence microscopy. Scattered and granular fluorescence in the cytoplasm of infected cells was considered a positive reaction.

Focus reduction neutralization test (FRNT). Serially diluted rodent sera (50 µl) were mixed with an equal volume of stock virus, either HTNV or SEOV, containing 200 focus forming units (FFU)/50 µl and incubated at 37 C for 1 hr in a CO_2 incubator. The mixtures were then inoculated onto Vero E6 cell monolayers grown on 8-chamber slides. The slides were incubated at 37 C for 1 hr, and the inoculum was removed. The cells were overlayed with MEM (supplemented with 5% FCS) containing 1.5% carboxymethyl cellulose (CMC) and incubated in a CO_2 incubator at 37 C for 6 to 11 days. After incubation, the infected cells were washed with PBS four times, fixed with methanol, and air-dried. To visualize the foci of virus-infected cells, IFA was carried out. Mouse immune serum to HTNV or SEOV was added to the Vero E6 cells on the slides inoculated with HTNV or SEOV, respectively. After incubation for 1 hr at 37 C and three washes with PBS, FITC-conjugated antibody to mouse IgG (ICN Pharmaceuticals, Inc., Aurora, Ohio, U.S.A.) was applied. After incubation for 1 hr at 37 C, the foci were counted under a fluorescence microscope. The FRNT titer was defined as the highest dilution of the serum that reduced the number of foci by at least 80%.

Western blot (WB). Western blot was performed using the recombinant nucleocapsid protein (rNP) of PUUV as an antigen (Kariwa et al., in preparation for publication). The rNP of PUUV was separated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, Mass., U.S.A.). Mouse immune serum to PUUV was used as a positive control to detect antigen on the membrane. IFA-positive rodent sera were reacted with the membrane, and bound antibodies were detected with horseradish peroxidase-labeled protein G (Prozyme, Inc., Calif., U.S.A.). The peroxidase substrate, 4-chloro-1-naphthol, was used to visualize the bands.

Capture enzyme-linked immunosorbent assay (ELISA). Seropositive human sera from the individuals tested at the Japan Ground Self-defense Force were further examined by capture-ELISA, using baculovirus-expressed recombinant HTNV, SEOV, and DOBV nucleocapsid proteins (rNP) produced in insect cells (1). The Fab region of mouse monoclonal antibody E5/G6 was used as the capture antibody to exclude non-specific reactions. Ninety-six-well plates were coated with E5/G6 (2 µg/ml in PBS) at 4 C overnight. The plates were then incubated with the baculovirus-expressed rNP at 37 C for 1 hr. As a negative control antigen, we used Borna disease virus p24 expressed by the baculovirus system. Each well was then incubated with 1:400-diluted seropositive serum samples or seronegative sera (negative control) at 37 C for 1 hr. After incubation, goat anti-human IgG conjugated with alkaline phosphatase was applied to the wells and incubated at 37 C for 1 hr. Finally, pNPP substrate was added, the plate was held at room temperature for 30 min, and the optical density (OD) was measured at 405 nm. OD values exceeding the mean of the serum control wells plus twice the standard deviation were regarded as positive.

Polymerase chain reaction (PCR) and sequence analysis. Total RNA was extracted from the lungs of seropositive *R. rattus* and *R. norvegicus* with Isogen reagent (Nippon Gene Co., Ltd., Osaka, Japan), according to the manufacturer's instructions. cDNA was synthesized using 5 µg of total RNA, Superscript II (200 U), and random primer (Invitrogen, Carlsbad, Calif., U.S.A.) incubated at 42 C for 50 min and at 70 C for 15 min. Partial S segments were amplified using the primer pair SEO-66 (5′-GAGAGAAATCAGTGCTCACG-3′) and SEO-801 (5′-ATAAACTCCCGGCAATAAGA -3′) and further amplified by an inner primer pair specific for SEOV, SEO-96 (5′-TGTGATAGCACGCCAGAAGG-3′) and SEO-542 (5′-TCCTCATATGAGCTGTCATC-3′). The polymerase chain reaction (PCR) program consisted of 35 cycles of denaturation at 94 C for 30 sec, annealing at 52 C for 30 sec, and extension at 68 C for 2 min. The amplified S segments were sequenced directly using a Big Dye terminator (Applied Biosystems, Foster City, Calif., U.S.A.) and an ABI 310 Genetic Analyzer.

Results

Epizootiological studies of hantavirus infection among wild rodents were conducted in various locations of Japan including the 4 major islands, i.e., Honshu, Kyushu, Shikoku, and Hokkaido from 2000 to 2003.

A total of 806 rodents and insectivores were captured from 11 wild settings on Honshu, Shikoku, Kyushu, and Tsushima Islands and from six sites on Hokkaido. Figure 1 shows the geographical locations of the survey sites. The sera were screened by IFA for anti-hantavirus antibodies. A total of 592 rodent sera from wild settings in the southern regions of Japan, including

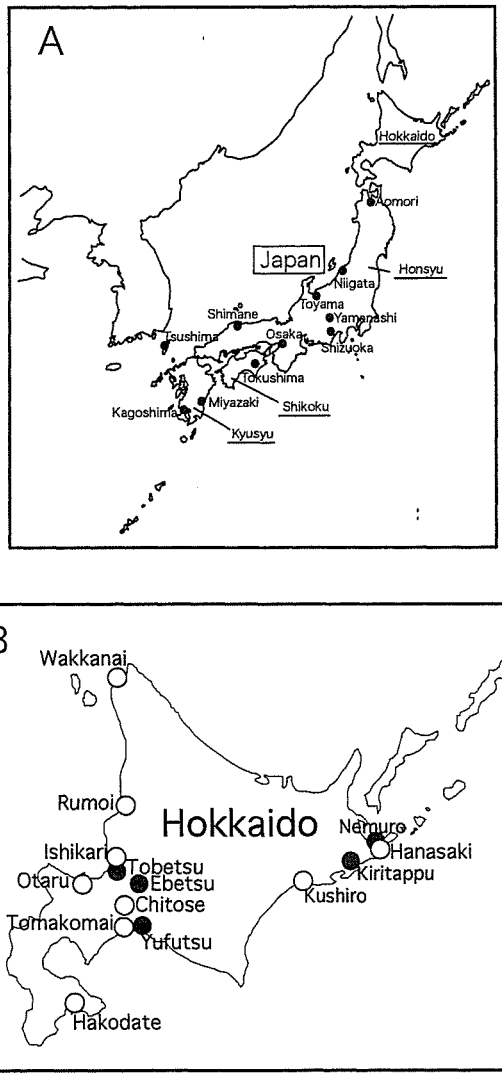

Fig. 1. Geographical location of epizootiological survey sites. (1) Surveys were carried out in Aomori, Niigata, Toyama, Yamanashi, Shizuoka, Osaka, Tokushima, Shimane, Tsushima, Miyazaki, and Kagoshima prefectures. (2) Surveys were carried out in Wakkanai, Rumoi, Ishikari, Otaru, Tomakomai, Hakodate, Tobetsu, Ebetsu, Chitose, Yufutsu, Kushiro, Kiritappu, Hanasaki, and Nemuro on Hokkaido, the northernmost main island of Japan. Open circles represent survey sites in seaports and airports; closed circles represent survey points of wild settings like forests.

Honshu, Shikoku, Kyushu, and Tsushima Islands, were screened for anti-hantavirus antibodies to HTNV, SEOV, and PUUV. Seropositive animals were detected in Toyama and Shimane (Table 1). Of 471 *A. speciosus*, 5 (1.1%) were seropositive by IFA. Two *R. norvegicus* (5.1%) from Toyama were positive for SEOV. No antibodies to PUUV were detected in any rodent species in the southern region of Japan. The IFA titers of seropositive *A. speciosus* to HTNV (1:32 to 1:128) were 4- to 8-fold higher than the titers to SEOV (1:16 to 1:64).

Table 1. Serological screening for 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]	0/14[b]	0/6	**4**/223	0/7	0/19	**1**/69	0/2	0/69	0/33	0/1	0/28	**5/471** (1.1)	4/471 (0.84)	0/471 (0)
A. argentius	—	—	0/9	0/7	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. dsinezumi	—	—	—	—	—	—	—	0/1	—	—	—	0/1 (0)	0/1 (0)	0/1 (0)
U. tapoides	—	—	—	—	—	—	—	0/5	—	—	—	0/5 (0)	0/5 (0)	0/5 (0)
Total	0/14	0/6	**6**/293	0/14	0/28	**1**/69	0/2	0/92	0/45	0/1	0/28	7/592(1.2)	**6**/592(1.0)	0/592 (0)

[a] Seropositive rodent species, the places where seropositive rodents were found, and the number of seropositive rodents are shown in bold face.
[b] Number of seropositive rodents/number of captured rodents.

Table 2. IFA and FRNT for seropositive rodents in Toyama and Shimane (2000–2003)

Place of survey	No. of positives	Species	IFA titer			FRNT[a]	
			HTNV	SEOV	PUUV	HTNV	SEOV
Toyama	4 /223[b]	*A. speciosus*	128	16	<16	20	<20
		A. speciosus	64	16	<16	20	<20
		A. speciosus	32	<16	<16	<20	<20
		A. speciosus	128	16	<16	<20	<20
	2/39	*R. norvegicus*	16	32	<16	NT	NT
		R. norvegicus	16	32	<16	NT	NT
Shimane	1 /69	*A. speciosus*	64	64	<16	<20	<20
Total							

[a] FRNT titer was expressed as a reciprocal of the highest dilution which showed 80% or more inhibition of the virus focus formation.

[b] Number of seropositive rodents/number of captured rodents.

Table 3. Serological screening for antibodies to hantavirus (HTN, SEO, PUU) by IFA among rodents captured in field surveys in Hokkaido (2000–2003)

Rodent species	Place of survey					Positive rate (%) to HTNV, SEOV, or PUUV
	Nemuro	Kiritappu	Ebetsu	Tobetsu	Yufutsu	
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 captured rodents is shown in parenthesis.

Seropositive *R. norvegicus* had slightly higher IFA titers to SEOV (1:32) than to HTNV (1:16) (Table 2). Some of the IFA-positive sera from *A. speciosus* neutralized HTNV (1:20) but not SEOV. Virus gene detection was attempted by RT-PCR in seropositive *A. speciosus*, but none of the samples showed the virus-specific PCR product bands (data not shown).

In contrast, of 214 animals captured in wild settings of Hokkaido, only 5 out of the 153 (3.26%) *C. rufocanus* were found to be seropositive in Nemuro, Ebetsu, and Tobetsu (Table 3). No other rodent species captured in wild settings had antibodies to hantaviruses. In rodents captured in urban or semi-urban settings, namely ports and an airport of Hokkaido, 4.5% (2/44) of *C. rufocanus*, 0.62% (2/321) of *R. norvegicus*, and 6.7% (3/45) of *R. rattus* were seropositive (Table 4). *C. rufocanus* captured in both wild settings and at the Chitose Airport had IFA titers to PUUV ranging from 1:32 to 1:128, while the titers to HTNV were negative or lower than those to PUUV. However, none of these serum samples had detectable levels of antibodies to SEOV. In addition, some positive sera from *C. rufocanus* were subjected to WB analysis with hantavirus rNPs to confirm the specific reactivity of the antibodies. All IFA-positive sera showed specific reactions with rNP, while no bands were observed in WB with IFA-negative sera (data not shown). Seropositive *R. norvegicus* and *R. rattus* were found in the port area of Rumoi, Otaru, and Hakodate. The IFA titers to SEOV and HTNV in these samples were almost equivalent, but antibodies to PUUV were below detectable levels (Table 5). In addition, lung tissues from seropositive *R. norvegicus* and *R. rattus* were subjected to RT-PCR to amplify the viral S segment. The partial S segment (256 nt) was amplified from one seropositive *R. rattus* captured at Hakodate that had a high IFA titer (1:512) to SEOV. The nucleotide sequence of the amplicon showed 96% identity with the SEOV prototype strain SR-11 (data not shown).

In order to examine the epidemiology of human hantavirus infection in an at-risk population, we screened sera from the personnel of the Japan Ground Self-defense Force on Hokkaido. Of 207 blood samples examined, one sample was positive (0.48%) for anti-

Table 4. Serological screening for antibodies to hantavirus (HTN, SEO, or PUU) 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	Wakkanai	Tomakomai	HTNV	SEOV	PUUV
C. rufocanus	2/39					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	0/12	1/115	0/28	1/56	0/4	0/9	0/3	0/35	0/59	2/321 (0.62)	2/321 (0.62)	0/321 (0)
R. rattus		2/44	1/1							3/45 (6.7)	3/45 (6.7)	0/45 (0)
Total	2/56	3/159	1/29	1/56	0/4	0/14	0/3	0/35	0/59	7/415 (1.7)	5/415 (1.2)	2/415 (0.48)

[a] Airport.

Table 5. Serological confirmation of hantavirus infection in seropositive rodents in Hokkaido (2000–2003)

Type of survey point	Place of survey	Animal number	Rodent species	Positives by IFA			WB	Nested-PCR	FRNT to SEOV
				HTNV	SEOV	PUUV			
Wild setting	Nemuro	N126	*C. rufocanus*	64	<16	128	(+)	NT	NT
	Ebetsu, Ishikari	E36	*C. rufocanus*	<16	<16	32	(+)	NT	NT
		E43	*C. rufocanus*	<16	<16	32	(+)	NT	NT
		E55	*C. rufocanus*	<16	<16	64	(+)	NT	NT
	Tobetsu	T37	*C. rufocanus*	<16	<16	128	NT	NT	NT
Airport and seaports	Chitose[a]	C36	*C. rufocanus*	32	<16	64	(+)	NT	NT
		C40	*C. rufocanus*	32	<16	64	(+)	NT	NT
	Rumoi[b]	R-9	*R. norvegicus*	64	64	<16	NT	(−)	<10
	Otaru[b]	13	*R. norvegicus*	64	64	<16	NT	(−)	<10
		15	*R. rattus*	64	64	<16	NT	(−)	<10
		114	*R. rattus*	128	256	<16	NT	(−)	<10
	Hakodate[b]	NH3	*R. rattus*	256	512	<16	NT	(+)	20

[a] Chitose Airport.
[b] Seaport.
NT: not tested.

Table 6. ELISA and WB for anti-hantaviral 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	128	256	<32
		Control	0.023	0.018	(−)	(−)	<32	<32	<32

NT: not tested.

hantavirus antibody by IFA, with titers of 1:256 to SEOV and 1:128 to HTNV. In order to confirm this finding, we carried out ELISA and WB analysis on the IFA-positive sample (Table 6). For the ELISA, various rNPs were used as antigens to determine the type of infecting virus (Fig. 2). The IFA-positive serum reacted most strongly with recombinant SEOV NP (rNP-SEO50) in the ELISA test. The reaction pattern was similar to that of a confirmed SEOV-infected patient serum (Fig. 2).

Discussion

Despite being surrounded by countries endemic for HFRS, Japan has not had a reported case of HFRS for about 20 years. During this period, however, anti-hantavirus antibodies have been detected in *R. norvegicus* captured in various Japanese ports. Our previous epizootiological surveys revealed that 10% of *C. rufocanus* on Hokkaido had anti-hantavirus antibodies and that this species carried PUUV-related viruses (6, 8). How-

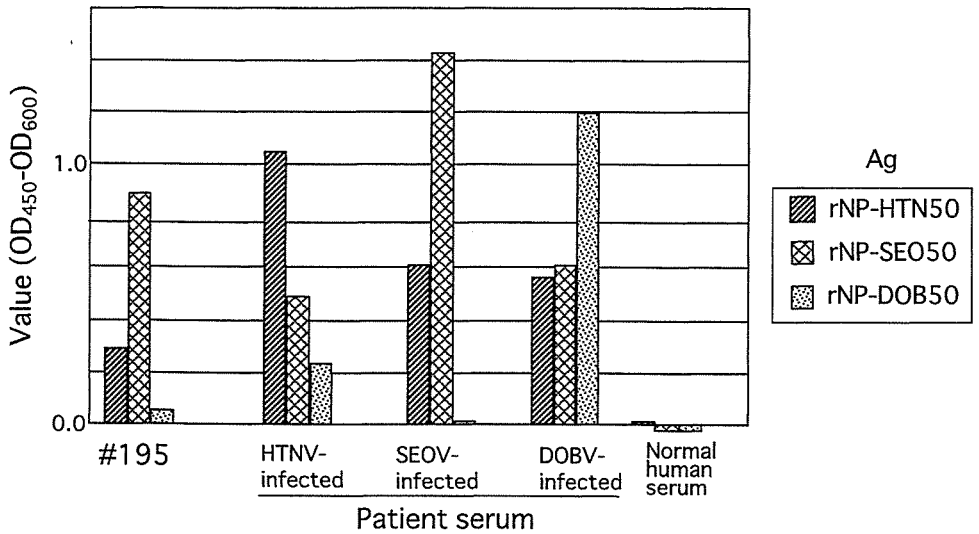

Fig. 2. Anti-hantavirus antibody detection by ELISA. IFA-positive human serum was incubated on a plate coated with different recombinant hantavirus NP with serotype-specific antigenic sites. The plate was incubated with goat anti-human IgG conjugated with alkaline phosphatase. Optical density (OD) was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

Table 7. Seroprevalence of hantavirus antibodies in rodents and insectivores of Japan (2000–2003)

Species	Number of seropositive	Number of captured	Positive rate (%)
A. speciosus	5	482	5/482 (1.0)
C. rufocanus	7	197	7/197 (3.6)
R. norvegicus	4	364	4/364 (1.1)
R. rattus	3	45	3/45 (6.7)
A. argenteus	0	59	0/59 (0)
A. peninsulae	0	4	0/4 (0)
C. rutilus	0	22	0/22 (0)
E. smithi	0	11	0/11 (0)
M. minutus	0	1	0/1 (0)
M. montebelli	0	11	0/11 (0)
M. musculus	0	4	0/4 (0)
C. dsinezumi	0	1	0/1 (0)
S. caecutiens	0	3	0/3 (0)
S. gracillium	0	11	0/11 (0)
S. unguiculatus	0	1	0/1 (0)
U. talpoides	0	5	0/5 (0)
Total	19	1,221	19/1,221 (1.6)

ever, epizootiological information on hantavirus infections in indigenous rodents in the southern parts of Japan has been extremely limited. Therefore, this study was carried out to elucidate whether rodent species other than *R. norvegicus* and *C. rufocanus* carry hantaviruses or antibodies to hantaviruses and to examine the prevalence of human infections in a high-risk group, such as personnel of the Japan Self-defense Force.

We found seropositive *A. speciosus* in Toyama and Shimane by IFA screening, as was the case in our previous study (2). In seropositive samples, the IFA titers to HTNV were 4- to 8-fold higher than the titers to SEOV, and the IFA titers to PUUV were all below 1:16. Some of the positive sera from *A. speciosus* neutralized HTNV but not SEOV (Table 2). These findings indicate that *A. speciosus* might carry hantaviruses that are more closely related to HTNV than to SEOV.

We were unable to amplify the virus gene from the seropositive *A. speciosus*. It is possible that the virus was present in seropositive animals at copy numbers too low to permit detectable amplification, or that the primers used in the PCR reaction had insufficient homology with the infecting virus to anneal. Further epizootiological surveys should be conducted to reveal what type of hantavirus is carried by *A. speciosus*. In addition, seropositive *R. rattus* and *R. norvegicus* found

in seaports and at the Chitose Airport could be sources of human SEOV infection, and their presence could pose a threat to people working in these facilities, to travelers, and to quarantine office employees. A higher seroprevalence was reported in workers employed in a reclaimed area where seropositive urban rats were detected (21). Therefore, a larger-scale epidemiological study of hantavirus infection among people associated with the seaports and airports in Japan is warranted.

Further, we found one seropositive individual among the personnel of the Japan Self-defense Force on Hokkaido, as confirmed by IFA, WB, and ELISA. The ELISA result indicated that the person might 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.

In this study we detected seropositive animals in *A. speciosus* (5/482, 1.0%), *R. norvegicus* (4/364, 1.1%), *R. rattus* (3/45, 6.7%), and *C. rufocanus* (7/197, 3.6%) among 1,221 animals captured in various areas and settings of Japan (Table 7). These four rodent species may serve as the reservoir animal of hantavirus in the country.

The results of this study, combined with previous findings, suggest that the low occurrence of HFRS in Japan might be attributed 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* might be mild and easily misdiagnosed; (3) human infection with PUU-related virus from *C. rufocanus* occurs only rarely; (4) the prevalence of hantavirus infection in *A. speciosus* is low.

In this study, we found that *R. rattus* in Japan carries hantaviruses; however, the infecting virus has not been well characterized. Because all hantaviruses must be considered potential human pathogens, we are now analyzing the virus in greater detail.

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GEOGRAPHICAL DISTRIBUTION OF HANTAVIRUSES IN THAILAND AND POTENTIAL HUMAN HEALTH SIGNIFICANCE OF *THAILAND VIRUS*

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Abstract. Phylogenetic investigations, sequence comparisons, and antigenic cross-reactivity studies confirmed the classification of *Thailand virus* (THAIV) as a distinct hantavirus species. The examination of sera from 402 rodents trapped in 19 provinces of Thailand revealed that five greater bandicoot rats (*Bandicota indica*) and one lesser bandicoot rat (*B. savilei*) from four provinces were focus reduction neutralization test (FRNT) antibody-positive for THAIV. One of 260 patients from Surin province in Thailand (initially suspected of having contracted leptospirosis, but found to be negative) showed symptoms compatible with hemorrhagic fever with renal syndrome (HFRS). The serum of this patient showed high titers of hantavirus-reactive IgM and IgG. FRNT investigations confirmed virus-neutralizing antibodies against THAIV. These observations suggest that THAIV or THAI-like viruses occur throughout Indochina and may represent an additional causative agent of HFRS.

INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) is a rodent-borne viral zoonosis caused by certain members of the viruses in the genus *Hantavirus* of the family *Bunyaviridae*.[1] The hantaviral species that have been causally associated with HFRS are *Hantaan virus* (HTNV), *Seoul virus* (SEOV), and *Dobrava/Belgrade virus* (DOBV) that are carried by the members of the rodents in the subfamily *Murinae* (Old World rats and mice), while the *Puumala virus* (PUUV) is carried by the members of the rodents in the subfamily *Arvicolinae* (voles and lemmings) of the family *Muridae*. Other hantaviral species that are not known as the causative agents of HFRS include *Tula virus* (TULV) and *Topografov virus* (TOPV) in Europe, *Khabarovsk virus* (KHAV) in far east Russia, and *Prospect Hill virus* (PHV) in the United States that are carried by rodents in the subfamily *Arvicolinae*. The *Thailand virus* (THAIV) is the only hantavirus species carried by the rodent in the subfamily *Murinae* in Thailand. *Thottapalayam virus* (TPMV) is the only hantavirus isolated from mammals in the Insectivore in India.[2]

The species of hantaviruses isolated from the rodents in the same subfamily; HTNV, SEOV, DOBV, and THAIV from rodents of subfamily *Murinae* and PUUV, TULV, TOPV, KHAV, and PHV from rodents of subfamily *Arvicolinae*, showed strong antigenic cross reactivity defined by antibody binding assays such as IFA and ELISA. Neutralization test is required to serologically distinguish among hantavirus species originated from rodents classified to the same subfamily.[3]

The THAIV strain Thai749 was originally isolated by Elwell et al. (1985) from a greater bandicoot rats (*Bandicota indica*) trapped in the vicinity of a small farm village in the western province of Kanchanaburi,[4] Thailand. Subsequent phylogenetic studies based on the nucleotide sequence of M segment of THAIV revealed that the THAIV is placed at the position most closely related to SEOV and grouped with other viruses from rodents classified to *Murinae*.[5] Thai749 strain is antigenically distinct from other hantavirus species.[3] However, only part of the nucleotide sequence information in the S segment of the THAIV is available so far.[5] For further understanding of THAIV of the relationship among other hantaviruses, nucleotide sequence information of entire S segment as well as further antigenic characterization is required.

It has been well characterized that a single rodent species or phylogenetically closely related rodent species are the principal host of a single hantavirus species.[6] The rodent fauna of Thailand includes 35 murine species in 7 genera and 1 arvicoline species, *Eothenomys melanogaster*.[7] A previous seroepizootiologic study of hantavirus infection conducted at central, northeastern, and near Bangkok areas revealed that greater bandicoot rat as a main reservoir and several species of rice field rats such as *Rattus rattus*, *exulans*, and *losea* are also natural reservoirs to a lesser extent in Thailand.[4] To extend our knowledge of the geographical distribution and natural host association of the hantaviruses in Thailand, we have continued further seroepizootiologic study, particularly by including the THAIV as antigen for serological screening.

Although the hantavirus infection spread in various species of rodents and wider areas in Thailand, epidemiologic information regarding to the human infection with hantavirus is quite limited. Suputthamongkol et al.[8] reported the first clinical case report of hantavirus infection in Thailand. However, the causative hantavirus species was not further characterized in the report. Since the clinical symptoms of leptospilosis and other febrile illness are similar to HFRS, undiagnosed HFRS cases would be existing among the patient with febrile illnesses of unknown etiology in Thailand.

In this study, we have examined antigenic and genetic prop-

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