identified on the American continents (Jonsson et al., 2010; Mills et al., 2010), some of which are associated with HPS. In North America, the Sin Nombre (SN), New York (NY), Monongahela, Black Creek Canal (BCC), Bayou (BAY) viruses cause HPS and are carried by *Peromyscus maniculatus*, *Peromyscus leucopus*, *Peromyscus leucopus nubiterrae*, *Sigmodon hispidus*, *Oryzomys palustis*, and *S. hispidus*, respectively (Morzunov et al., 1995; Nichol et al., 1993; Ravkov et al., 1995; Rawlings et al., 1996; Song et al., 1994; Torrez-Martinez and Hjelle, 1995). In South America, the Andes (AND), Bermejo, Lechiguanas, Maciel, Oran (ORN), Laguna Negra, Araraquara, and Juquitiba viruses are associated with HPS and are carried by *Oligoryzomys longicaudatus*, *Oligoryzomys chacoensis*, *Oligoryzomys flavescens*, *Bolomys obscurus*, *O. longicaudatus*, *Calomys laucha*, *Bolomys lasiurus*, and *Oligoryzomys nigripes*, respectively (Bohlman et al., 2002; Calderón et al., 1999; Chu et al., 2006; Johnson et al., 1997; Padula et al., 2002; Suzuki et al., 2004). Other hantaviruses that are not associated with human disease have been identified in various rodents in South America (Jonsson et al., 2010; Mills et al., 2010) such as Rio Mamore virus which was first identified indigenous hantavirus in this geographical region (Bharadwaj et al., 1997)

In Central America, Choclo virus, carried by *Oligoryzomys fulvescens*, has been identified as a cause of HPS in Panama (Vincent et al., 2000). Other viruses, including Rio Segundo (RIOS), Catacamas (CAT), and Calabazo, have been identified in *Reithrodontomys mexicanus* in Costa Rica, *Oryzomys couesi* in Honduras, and *Zygodontomys brevicauda* in Panama, respectively; however, their pathogenicity in humans is unknown (Hjelle et al., 1995; Milazzo et al., 2006; Vincent et al., 2000). Although there have been no reported cases of HPS, serological studies in rodents have indicated that several hantaviruses exist in Mexico (Castro-Arellano et al., 2009; Hjelle et al., 1995; Mantooth et al., 2001; Suzán et al., 2001). The first hantavirus genome detected in Mexico was that of El Moro Canyon (ELMC) virus from *Reithrodontomys megalotis* in 1995 (Hjelle et al., 1995). Another hantavirus, Playa de Oro (ORO) virus, identified recently in *O. couesi* in Western Mexico is most related genetically with the CAT, BAY, and BCC viruses (Chu et al., 2008). Because of the strong diversity among rodent species in Mexico and the specific nature of the virus–rodent relationship, more unrecognized hantaviruses likely exist. The objective of this study was to extend our knowledge of hantavirus ecology in Mexico and to increase our understanding of hantavirus evolution in the Americas.

Here we report the identification of three distinct hantaviruses, whose provisional names are Montano (MTN) virus in *Peromyscus beatae*, Carrizal (CAR) virus in *Reithrodontomys sumichrasti*, and Huitzilac (HUI) virus in *R. megalotis* in the southern Mexican states of Guerrero and Morelos. The nucleotide sequences of the open reading frames of the S, M, and L genome segments of the three Mexican hantaviruses were determined and a genetic analysis was performed. Our findings provide insight into the evolution of hantaviruses on the American continents.

2. Materials and methods

2.1. Rodent survey

In May of 2006, wild rodents were captured using live traps placed amid vegetation in a tropical moist forest 2400–2600 m above sea level (asl) in Leonardo Bravo and in a dry forest around 1000–1400 m asl in Eduardo Neri, near Zumpango del Río, in the state of Guerrero. Rodents were also trapped at the Agricultural Experiment Station in Zacatepec (600–1000 m asl), and in a pine forest with grasses above 2850 m asl in the municipality of Huitzilac near Tres Marías, in the state of Morelos. A total of 211 rodents were captured at these survey points. Blood samples were collected from live animals via cardiac puncture under anesthesia, followed by the collection of lungs, kidneys, spleen, and liver. The collected organs were stored at −80 °C until use. Filter paper was used to collect blood samples from dead animals. The serum and clots were separated by centrifugation at 12,000 × *g* for 5 min. The sera were heat-inactivated at 56 °C for 30 min and stored at −40 °C until required.

2.2. ELISA

The construct for expression of recombinant N of SNV (SNV-rN) was kindly supplied by Dr. C.J. Peters (University of Texas Medical Branch, Galveston, TX, USA). SNV-rN was expressed in *Escherichia coli*, purified by Ni-column chromatography, and stored at −80 °C until required. SNV-rN was added to 96-well plates (50 μl/well) and kept at 4 °C overnight. The plates were washed six times with phosphate-buffered saline containing 0.05% Tween 20 (PBST), blocked with 3% bovine serum albumin (Nakalai, Kyoto, Japan) in PBST, and incubated at 37 °C for 1 h. After washing, 1:100 dilutions of wild rodent sera were applied to the plates (50 μl/well) and incubated at 37 °C for 1 h. The plates were washed and incubated with 50 μl/well of peroxidase (PO)-conjugated anti-*P. leucopus* IgG (H+L) antibodies (KPL, Inc., Gaithersburg, MD, USA) or PO-conjugated protein G (Zymed, San Francisco, CA, USA; 50 μl/well) at 37 °C for 1 h. After washing, 200 μl of O-phenylenediamine tablets (Sigma–Aldrich, St. Louis, MO, USA) with hydrogen peroxide was added to each well, and the plate was left at room temperature for 30 min. Optical density values were then measured at 450 nm using a spectrophotometer (Labsystem, Helsinki, Finland).

2.3. Western blotting

SNV-rN was diluted to 1 μg/ml in sample buffer [1% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 0.05% bromophenol blue, and 10% glycerol] and boiled for 1 h. SNV-rN was subjected to SDS–PAGE at 40 mA for 2 h, and then transferred to a polyvinylidene difluoride (PVDF) membrane in transfer buffer (0.1 M Tris, 0.192 M glycine, and 20% methanol) at 1 mA/cm^2 for 90 min using a semi-dry system. The membrane was cut to about 5 mm in width, immersed in BlockAce (Dai Nippon Pharmaceutical, Osaka, Japan) at 4 °C overnight, and washed four times with PBST. Rodent sera were diluted 1:400 in PBS and each was applied to the PVDF membrane. The membrane was then washed with PBST and immersed in a 1:5000 dilution of PO-conjugated anti-*Peromyscus* IgG (KPL, Inc.) at room temperature for 1 h. After washing, the membrane was soaked in ECL Detection Reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at room temperature for 1 min. Chemical luminescence was then detected using a CCD camera system (Aishin, Kariya, Japan).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

The lungs of the captured wild rodents were lyophilized in ISOGEN RNA extraction reagent (Nippon Gene, Tokyo, Japan) by shaking with zirconium beads at 3/s for 3 min, using a Qiagen M300 mixer (Retsch, Haan, Germany). After being kept at room temperature for 5 min, the lyophilized samples were stored at −80 °C until they were used for RNA extraction. Total RNA was extracted according to the manufacturer's instructions. RNA (2 μg) was first reverse-transcribed using 200 U of Superscript II RNase H-reverse transcriptase (Invitrogen Corp., San Diego, CA, USA) and 5 ng of random primers (Invitrogen Corp.) according to the manufacturer's instructions. Partial S genome segments were amplified using the cDNA, a forward primer, SN-S143Fw (5′-tgg acc cvg atg ayg tya aca

a-3′), a reverse primer, SN-S716Rv (5′-aan ccw ats ach ccc atg ac-3′), and Platinum Taq DNA Polymerase High Fidelity (Invitrogen Corp.). The thermal conditions for PCR were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min.

2.5. Sequencing of the hantavirus genome segments

The amplified DNA fragments were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) then sequenced using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. In some of the samples from which partial hantavirus S genome segments were amplified, the nucleotide sequences of all genome segments (S, M, and L) covering open reading frames were determined by direct sequencing of the amplified fragments.

2.6. Genetic analysis

The hantavirus nucleotide sequences used in this study were listed in supplemental Table S1 and were compared using Genetyx version 8.0.0 (Genetyx Corp., Tokyo, Japan) to calculate nucleotide and amino acid identities. A software package for genetic analysis, MEGA 4 (http://www.megasoftware.net/mega.html) was used to generate multiple alignments and phylogenetic trees by the neighbor-joining method. The reliability of the dendrogram was evaluated using 1000 bootstrap replicates.

3. Results

3.1. Investigation of hantavirus infection in Mexican rodents

To obtain epizootiological information on hantavirus infection, Mexican rodents were captured at four survey points, including two in Guerrero state (municipalities of Leonardo Bravo and Eduardo Neri) and two in Morelos state (municipalities of Zacatepec and Huitzilac) (Fig. 1 and Table 1). A total of 211 rodents were tested for anti-hantavirus antibodies and S genome segments. In Leonardo Bravo, 25 of 130 (19.2%) rodents were seropositive by ELISA, including 17 *P. beatae*, 1 *Megadontomys thomasi*, 1 *Neotoma picta*, and 6 *R. sumichrasti* (Table 1). No hantavirus-specific antibodies were detected in Eduardo Neri or Zacatepec. In Huizilac, antibodies were detected in two *R. megalotis* (Table 1). The S genes were amplified from 21 rodents, including 16 *P. beatae*, 1 *Peromyscus megalops*, 3 *R. sumichrasti*, and 1 *R. megalotis*.

3.2. Genetic analysis of hantaviruses

The nucleotide sequences of the entire coding regions of the hantavirus S, M, and L genes from one each of *P. beatae*, *R. sumichrasti*, and *R. megalotis* were determined. The hantaviruses identified from *P. beatae* at Leonardo Bravo, Guerrero State (17°36′13″N, 99°50′9″W), *R. sumichrasti* at Leonardo Bravo, Guerrero State (17°36′35″N, 99°49′15″W), and *R. megalotis* at Huitzilac, Morelos State (19°03′33.8″N, 99°14′58.9″W) were provisionally designated as Montano (MTN) [AB620100, AB620101, and AB620102], Carrizal (CAR) [AB620103, AB620104, and AB620105], and Huitzilac (HUI) [AB620106, AB620107, and AB620108], respectively (Table S1). The nucleotide sequence identities among these Mexican viruses were 77.9–84.1% (Table 2). The S gene of MTN had the highest nucleotide and amino acid identities with that of LSC virus at 80.2 and 94.2%, respectively. The nucleotide and amino acid identities of the S gene of MTN with the other New World hantaviruses were 73.5–78.6% and 82.4–90.4%, respectively. Although the S genes of the HUI and CAR viruses differed by about 16% at the nucleotide level, both were closely related to ELMC virus (83% nucleotide and 96.3–98.6% amino acid sequence identities). Interestingly, MTN, CAR, and HUI viruses from rodents of the family

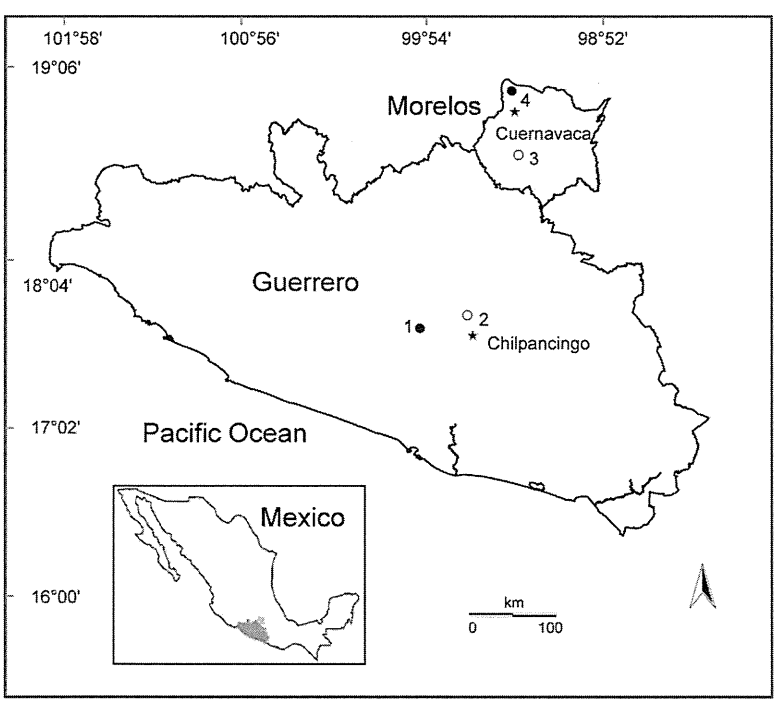

Fig. 1. Rodent survey points in Guerrero and Morelos, Mexico. Rodents were captured at two locations in Guerrero state, Leonardo Bravo (1) and Eduardo Neri (2), and at two locations in Morelos state, Zacatepec (3) and the municipality of Huitzilac, Tres Marías (4). Closed circles indicate locations where hantavirus-infected rodents were captured. Open circles indicate locations where no hantavirus-infected rodents were captured.

Table 1
Detection of anti-hantavirus antibodies and hantavirus RNA in Mexican rodents.

Species	No. of captured animals	No. of Ab-positive animals (%)		No. of virus RNA-positive animals by RT-PCR (%)
		ELISA (%)	WB	
Leonardo Bravo, Guerrero				
Baiomys musculus	1	0		0
Peromyscus beatae	50	17 (34.0)	16	16 (32.0)
Peromyscus lepturus	5	0		0
Peromyscus megalops	24	0		1 (4.2)
Peromyscus mexicanus	10	0		0
Peromyscus spp.	14	0		0
Megadontomys thomasi	6	1 (16.7)	1	0
Neotoma picta	6	1 (16.7)	1	0
Reithrodontomys sumichrasti	14	6 (42.9)	6	3 (21.4)
Subtotal	130	25 (19.2)	24	20 (15.4)
Eduardo Neri, Guerrero				
Hodomys alleni	4	0		0
Osgoodomys banderanus	1	0		0
Peromyscus melanophrys	3	0		0
Reithrodontomys sumichrasti	1	0		0
Subtotal	9	0		0
Zacatepec, Morelos				
Mus musculus	10	0		0
Liomys irroratus	2	0		0
Subtotal	12	0		0
Huitzilac and Tres Marías, Morelos				
Microtus mexicanus	15	0		0
Mus musculus	2	0		0
Neotoma mexicana	1	0		0
Neotomodon alstoni	2	0		0
Peromyscus hylocetes	13	0		0
Peromyscus maniculatus	2	0		0
Reithrodontomys megalotis	25	2 (8.0)	1	1 (4.0)
Subtotal	60	2 (3.3)	1	1 (1.7)
Total	211	27 (12.8)	25	21 (10.0)

Cricetidae, subfamily Neotominae, had only 76% nucleotide and 84% amino acid identities with ORO virus another Mexican hantavirus, which was identified from the family Cricetidae, subfamily Sigmodontinae, *O. couesi*, in Colima, Mexico. The S segments of the MTN, CAR, and HUI viruses encode an N protein of 428 amino acids, which is equivalent in length to those of other American hantaviruses, including ELMC, SN, and AND viruses. The nucleotide and amino acid sequences of the M segment from the MTN, CAR, and HUI viruses were more diverse at 72.4–80.4% and 80.8–93.0% identities, respectively (Table 3). In terms of known hantaviruses, the M gene of MTN virus had the highest nucleotide and amino acid identities with that of LSC virus at 76.1 and 88.9%, respectively. The M genes of the HUI and CAR viruses had the highest identities with those of ELMC virus at ~78% at the nucleotide level and 90–91% at the amino acid level. Mexican virus M genes had 70–74% nucleotide and 73–83% amino acid identities with other known American hantaviruses. The M segments of the MTN, CAR, and HUI viruses encode a glycoprotein precursor (GPC) of 1139 amino acids, while the S segments of the SN, ELMC, and AND viruses encode GPCs of 1140, 1139, and 1138 amino acids, respectively. The number of cysteine residues in the GPCs of the MTN, CAR, and HUI viruses was 62, 62, and 63, respectively, while those of SN, ELMC, and AND were 61, 62, and 64, respectively. Four possible glycosylation sites in GPC, three in Gn and one in Gc, were conserved in the MTN, CAR, and HUI viruses. A possible signal peptidase cleavage motif "WAASA" (Lober et al., 2001) was present in the Mexican hantaviruses. A similar genetic relationship was observed between the L genes of the Mexican and other hantaviruses (Table 4). The L protein of the Mexican and all other American hantaviruses consisted of 2153 amino acids. Amino acid residues conserved in negative-stranded RNA viruses (Kukkonen et al., 2005) were also conserved in the L proteins of the MTN, CAR, and HUI viruses (data not shown).

3.3. Phylogenetic analysis of Mexican hantaviruses

A phylogenetic analysis of the S and M segments revealed that the Mexican hantaviruses occupied a monophyletic clade with the LSC, ELMC, and RIOS viruses (Figs. 2 and 3). The lineage of Mexican hantaviruses including MTN, CAR, and HUI viruses was apparently distinct from other lineages, such as SN–NY, ORO–BAY–BCC, and South American hantaviruses. A similar phylogeny was also observed for the L segment (Fig. 4).

4. Discussion

More than 30 hantaviruses have been identified in North and South America (Jonsson et al., 2010; Mills et al., 2010) since HPS was first reported in 1993 in the US (Nichol et al., 1993; Macneil et al., 2011). Many American hantaviruses cause HPS and as such represent threats to human health. In Mexico, however, there have been no reported HPS cases, although hantavirus genomes have been detected in several rodent species, including ELMC virus from *R. megalotis* (Hjelle et al., 1995) and ORO virus from *O. couesi* (Chu et al., 2008). In addition, anti-hantavirus antibodies have been detected in *P. maniculatus*, *R. sumichrasti*, *Peromyscus levipes*, *Peromyscus hylocetes*, *Peromyscus melanotis*, and *Sigmodon mascotensis*

Table 2
Nucleotide and amino acid identities of S segment entire coding region among newly recognized Mexican and other hantaviruses.

	MTN	HUI	CAR	ELMC	LSC	RIOS	SN	NY	ORO	BAY	BCC	AND	LN	ORN	PH	PUU	HTN	SEO
Nucleotide identity (%)																		
MTN	–	77.9	78.9	78.6	80.2	77.0	75.2	76.6	75.8	73.5	74.9	75.7	74.9	74.5	70.6	69.8	63.1	64.1
HUI	91.4	–	84.1	82.9	79.5	78.9	76.1	75.2	75.8	75.4	75.4	75.9	74.2	75.4	68.8	68.0	64.4	64.7
CAR	91.4	96.7	–	82.7	79.2	79.0	76.1	77.0	76.4	76.1	74.8	75.7	75.2	75.9	69.1	68.3	62.8	62.9
ELMC	90.4	98.6	96.3	–	80.5	91.1	76.6	76.9	76.6	76.6	75.6	76.7	75.1	77.9	71.5	68.5	64.3	65.0
LSC	94.2	91.7	91.2	91.0	–	89.2	77.0	76.9	76.9	74.5	75.6	75.6	74.7	76.8	70.7	67.9	63.1	64.4
RIOS	89.0	91.6	91.6	79.1	78.3	–	75.4	73.7	75.9	74.7	73.5	74.5	75.1	75.6	70.1	70.2	62.1	62.6
SN	86.9	84.6	84.6	84.6	85.2	83.2	–	83.4	76.4	76.9	75.4	76.2	76.4	77.1	70.2	68.3	63.1	64.1
NY	86.2	84.8	84.6	84.3	85.7	82.9	93.7	–	78.0	77.6	76.0	77.3	76.5	77.1	71.1	69.6	62.3	63.8
ORO	84.1	84.1	83.9	84.1	84.2	83.4	85.5	86.4	–	80.4	79.4	76.9	77.4	78.6	71.7	68.9	64.8	63.6
BAY	85.7	84.1	83.4	84.6	84.4	83.6	86.9	87.6	92.8	–	80.8	76.9	77.5	78.4	72.3	70.3	65.3	62.5
BCC	83.9	83.4	82.9	83.4	83.2	82.0	83.9	85.5	89.7	92.3	–	76.5	77.0	76.4	71.0	68.3	64.6	64.5
AND	84.6	83.2	82.9	82.9	84.4	81.8	86.0	87.6	87.6	88.3	86.4	–	79.2	83.7	71.3	68.1	65.3	63.4
LN	82.5	82.5	83.4	82.2	82.7	80.6	85.3	87.2	87.9	87.2	85.5	90.4	–	79.3	70.0	70.1	66.1	65.4
ORN	84.1	82.9	83.2	82.9	83.2	81.5	86.9	87.6	88.1	89.0	86.4	96.7	90.2	–	72.5	68.7	64.6	65.0
PH	76.0	75.3	74.1	74.8	76.4	73.2	74.1	76.2	76.2	77.4	77.1	76.0	75.3	75.5	–	72.2	64.9	64.4
PUU	70.7	70.4	71.1	70.2	70.2	70.9	70.7	71.4	72.5	72.7	73.7	72.5	71.8	72.1	79.7	–	62.8	62.3
HTN	62.2	62.5	62.7	62.0	61.2	62.0	62.5	63.2	62.7	63.9	64.3	64.8	64.3	65.3	62.1	60.5	–	75.2
SEO	63.2	62.0	61.1	61.5	62.2	60.8	62.0	63.4	63.2	63.2	63.9	64.3	63.2	64.1	63.5	61.9	82.8	–
Amino acid identity (%)																		

Table 3
Nucleotide and amino acid identities of M segment entire coding region among newly recognized Mexican and other hantaviruses.

	MTN	HUI	CAR	ELMC	LSC	SN	NY	BAY	BCC	AND	LN	ORN	PH	PUU	HTN	SEO
Nucleotide identity (%)																
MTN	–	72.4	73.4	71.6	76.1	72.9	73.1	71.6	71.5	70.0	69.4	69.6	66.2	65.4	60.9	59.8
HUI	81.1	–	80.4	78.7	74.2	72.9	72.9	71.5	72.1	70.5	70.3	70.0	66.5	66.6	61.0	61.3
CAR	80.8	93.0	–	78.1	74.0	72.6	73.7	71.4	71.6	70.6	70.4	70.1	65.8	66.7	61.4	62.2
ELMC	79.7	91.0	90.2	–	72.8	72.1	71.8	70.8	71.4	69.1	70.0	69.3	66.2	65.9	61.3	61.2
LSC	88.9	83.4	82.6	80.3	–	74.2	73.8	72.3	72.9	71.6	70.4	70.2	66.9	66.8	61.3	60.6
SN	82.6	82.0	80.5	79.7	83.3	–	81.0	72.9	73.5	71.9	71.3	71.4	67.1	66.1	60.6	59.4
NY	82.3	81.1	80.1	79.3	83.6	95.7	–	72.5	72.8	71.8	72.2	70.7	67.4	67.3	60.8	59.3
BAY	78.5	78.3	77.7	76.5	79.0	80.8	79.6	–	88.5	71.1	70.3	70.4	65.4	64.7	59.9	60.2
BCC	77.6	78.3	77.0	76.4	79.0	80.2	79.6	77.7	–	71.5	71.4	70.9	64.6	66.2	60.4	60.8
AND	74.2	76.2	75.3	73.7	75.8	78.4	77.7	76.0	75.5	–	75.6	79.0	65.1	65.7	59.8	59.5
LN	72.9	75.3	75.0	74.1	75.5	76.8	76.5	75.8	75.7	86.8	–	75.9	66.4	65.6	60.2	59.4
ORN	74.3	75.1	75.4	72.9	75.5	77.7	77.6	75.5	75.6	92.3	86.3	–	65.3	64.9	59.4	59.0
PH	67.3	68.3	68.7	66.8	68.1	67.6	67.8	66.3	65.4	67.2	66.3	66.9	–	70.6	60.3	59.5
PUU	66.3	67.4	67.6	66.5	67.1	67.0	67.2	64.6	65.5	67.7	65.9	67.2	75.5	–	60.4	60.5
HTN	57.0	57.0	57.0	56.6	58.2	55.0	54.6	54.8	54.1	55.2	54.3	54.9	54.2	54.8	–	72.3
SEO	55.2	55.7	55.6	55.0	56.1	53.2	53.3	54.7	53.7	54.2	54.0	54.7	54.1	53.8	76.9	–
Amino acid identity (%)																

Table 4
Nucleotide and amino acid identities of L segment entire coding region among newly recognized Mexican and other hantaviruses.

	MTN	HUI	CAR	SN	CC	AND	PH	PUU	HTN	SEO
					Nucleotide identities (%)					
MTN	–	75.4	77.0	75.6	76.2	74.5	70.7	71.8	66.5	67.3
HUI	86.4	–	94.4	75.2	75.8	74.5	70.7	71.5	67.3	67.2
CAR	86.8	80.0	–	76.2	75.6	75.0	71.0	71.8	67.0	67.1
SN	86.9	87.6	87.3	–	87.9	75.5	70.4	71.2	66.7	66.9
CC	86.9	87.6	87.3	99.1	–	74.9	70.8	72.2	66.6	67.2
AND	84.2	84.6	85.0	86.8	86.7	–	70.2	71.5	66.7	66.5
PH	77.8	78.4	78.6	78.7	78.6	77.7	–	72.8	65.5	66.4
PUU	77.0	77.6	77.9	77.8	77.8	77.3	82.8	–	68.9	67.5
HTN	68.7	69.2	69.3	69.2	69.3	68.6	67.9	66.8	–	74.2
SEO	69.0	68.4	68.9	69.0	69.1	68.1	68.3	68.4	85.0	–
					Amino acid identities (%)					

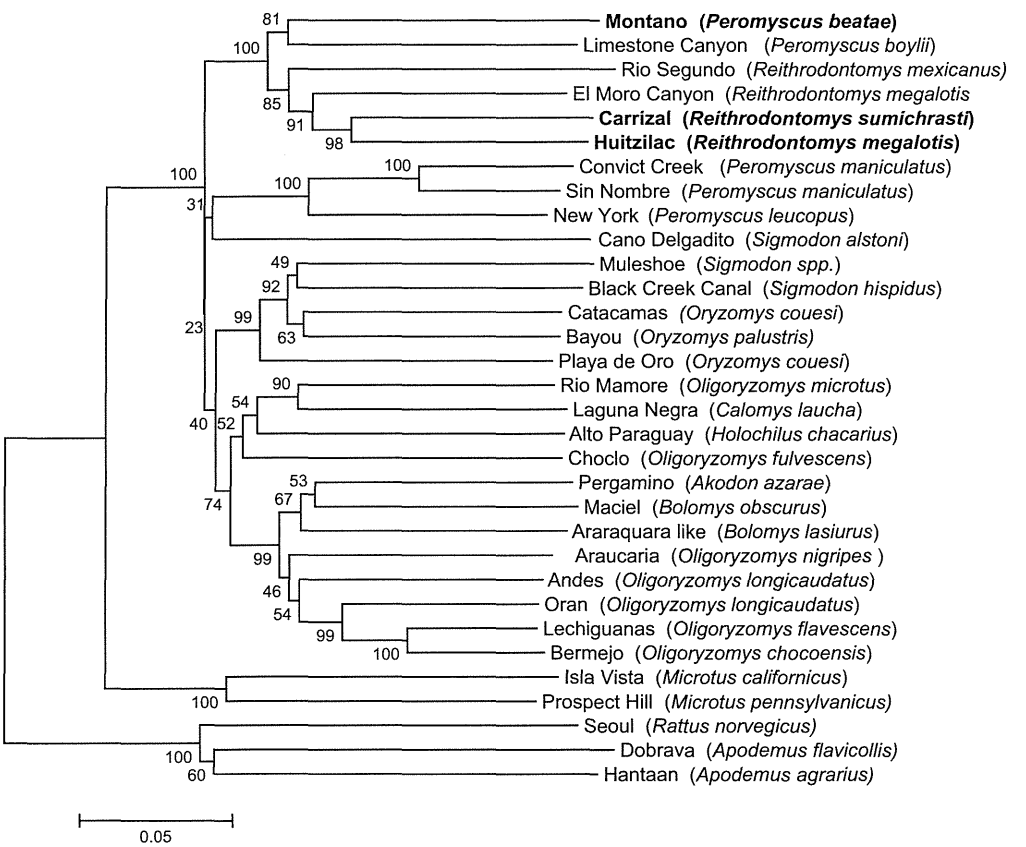

Fig. 2. Phylogenetic tree of the hantavirus S gene. Hantavirus S genes (from 43 to 1329 nucleotides corresponding to Sin Nombre virus) were used to construct the phylogenetic tree. A software package for genetic analysis, MEGA 4 (http://www.megasoftware.net/mega.html), was used to generate multiple alignments; the phylogenetic tree was drawn by the neighbor-joining method. Horizontal distances are proportional to the minimum number of nucleotide differences. The reliability of the dendrogram was evaluated by 1000 bootstrap replicates. The numbers beside the branches indicate bootstrap values expressed as percentages. The academic name in parentheses is the primary rodent host of the virus.

(Castro-Arellano et al., 2009; Hjelle et al., 1995; Mantooth et al., 2001; Suzán et al., 2001). Mexico is a mosaic of different environmental conditions that likely led to evolution in a large variety of habitats. The ecology of rodent species is extremely complex in Mexico; thus, more hantaviruses may be discovered. Therefore, it is important to investigate these viruses and their rodent hosts from the viewpoint of hantavirus evolution in North and South America, as well as their importance for public health.

In this study, we detected hantavirus infection in five rodent species, including *P. beatae*, *M. thomasi*, *N. picta*, *R. sumichrasti*, and *R. megalotis* in the Mexican states of Guerrero and Morelos (Table 1). MTN virus had the closest relationship with LSC virus, which is carried by *Peromyscus boylii* (Tables 1 and 2 and Figs. 2 and 3). Interestingly, these *Peromyscus*-borne viruses were more related to *Reithrodontomys*-borne viruses, including RIOS virus (*R. mexicanus*) and ELMC virus (*R. megalotis*), than to other *Peromyscus*-borne viruses such as SN and NY. Our results support the previous finding that LSC virus is more closely related to *Reithrodontomys*-borne viruses than to other *Peromyscus*-borne viruses (Sanchez et al., 2001). In this study, we also identified CAR and HUI viruses in *R. sumichrasti* and *R. megalotis*, respectively, in Mexico. These viruses occupied a monophyletic clade with other *Reithrodontomys*-borne ELMC and RIOS viruses (Figs. 2 and 3). Therefore, it is possible that an ancestral Peromyscine species of

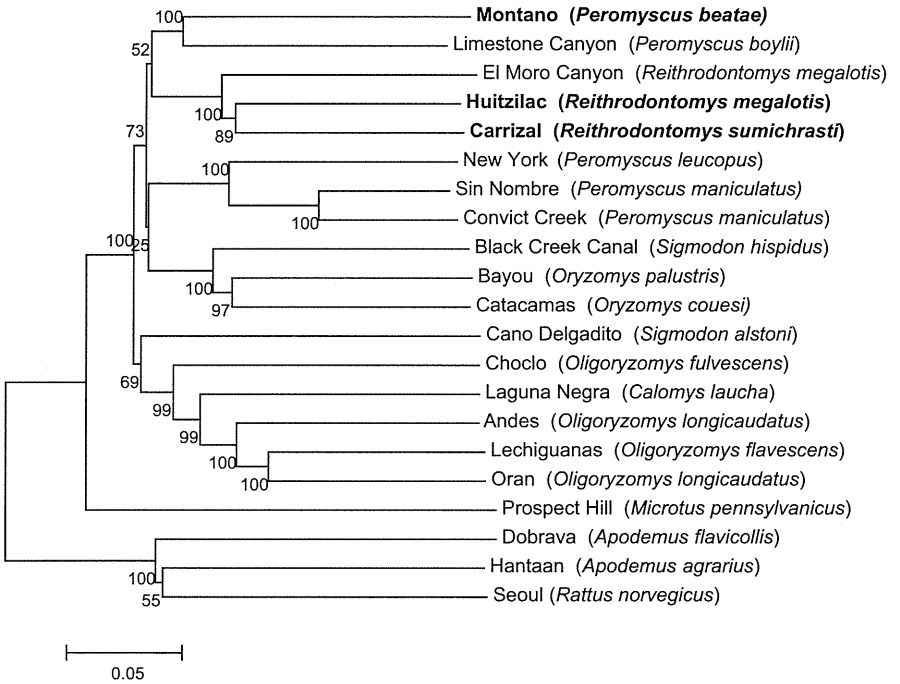

Fig. 3. Phylogenetic tree of the hantavirus M gene. Hantavirus M genes (from 52 to 3474 nucleotides corresponding to Sin Nombre virus) were used to construct the phylogenetic tree. A software package for genetic analysis, MEGA 4 (http://www.megasoftware.net/mega.html), was used to generate multiple alignments; the phylogenetic tree was drawn by the neighbor-joining method. Horizontal distances are proportional to the minimum number of nucleotide differences. The reliability of the dendrogram was evaluated by 1000 bootstrap replicates. The numbers beside the branches indicate bootstrap values expressed as percentages. The academic name in parentheses is the primary rodent host of the virus.

P. beatae and *P. boylii* was infected with a *Reithrodontomys*-borne hantavirus and virus–rodent co-speciation then occurred. Although co-speciation is considered to be the main driver of hantavirus evolution, host-switching events have been suggested for various hantaviruses (Arai et al., 2008; Kang et al., 2011; Morzunov et al., 1998; Nemirov et al., 2002; Plyusnina et al., 2009; Wang et al., 2000). To obtain conclusive data regarding hantavirus host switching among *Reithrodontomys* and *Peromyscus* rodents, further study of both hantavirus genes and rodent DNA such as cytochrome B gene is required.

MTN and CAR viruses were identified from *P. beatae* and *R. sumichrasti*, respectively, which is the first time that hantavirus genomes have been detected in these species. HUI virus was detected in *R. megalotis*, in which ELMC virus had already been identified (Rowe et al., 1995; Torrez-Martinez et al., 1995). However, the identification of different hantaviruses from the same rodent species in different subspecies or different regions occurred, such

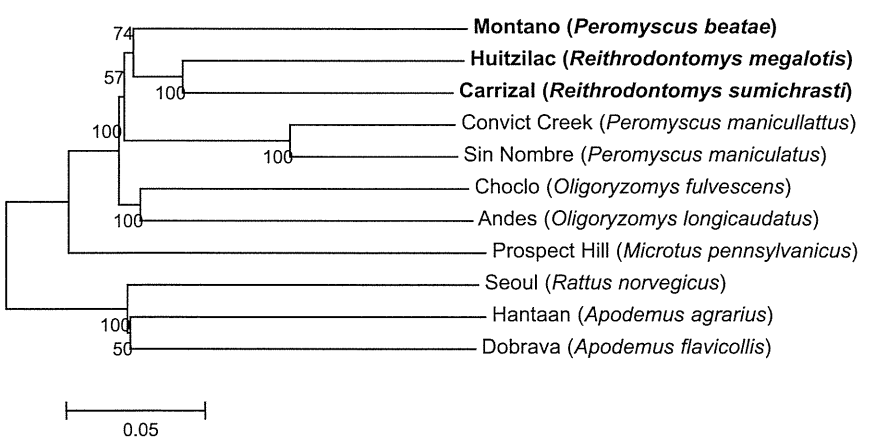

Fig. 4. Phylogenetic tree of the hantavirus L gene. Hantavirus L genes (from 36 to 6497 nucleotides corresponding to Sin Nombre virus) were used to construct the phylogenetic tree. A software package for genetic analysis, MEGA 4 (http://www.megasoftware.net/mega.html), was used to generate multiple alignments; the phylogenetic tree was drawn by the neighbor-joining method. Horizontal distances are proportional to the minimum number of nucleotide differences. The reliability of the dendrogram was evaluated by 1000 bootstrap replicates. The numbers beside the branches indicate bootstrap values expressed as percentages. The academic name in parentheses is the primary rodent host of the virus.

as ORO and CAT viruses in *O. couesi*, SN and Monongahela viruses in *P. maniculatus*, AND and ORN viruses in *O. longicaudatus*, and Hantaan and Saaremaa viruses in *Apodemus agrarius* (Chu et al., 2008; Lee et al., 1978; Levis et al., 1998; López et al., 1996; Milazzo et al., 2006; Nemirov et al., 1999; Nichol et al., 1993; Song et al., 1996). Therefore, it is possible that *R. megalotis* may be the host species of HUI virus. However, to obtain more conclusive data on host species of Mexican hantaviruses further epizootiological surveys are required.

The MTN, CAR, and HUI viruses are distant from ORO virus, which has been identified in *O. couesi* in Mexico. ORO virus occupies a monophyletic clade with hantaviruses associated with North American Sigmodontinae rodents, including BAY and BCC. In contrast, the MTN, CAR, and HUI viruses are associated with Neotominae rodents. The genetic distance between the three Mexican viruses and ORO may reflect the long history of hantavirus evolution in two separate rodent subfamilies, Neotominae and Sigmodontinae.

It is important to know whether hantavirus infection exists in Mexican people, though no HPS cases have been reported so far. It is evident that five hantaviruses, ELMC, ORO, MTN, CAR, and HUI, are circulating in Mexican rodents. Furthermore, *P. maniculatus*, *P. leucopus*, and *S. hispidus* are the hosts of the pathogenic SN, NY, and BCC viruses, respectively, in the US and these species are also widely distributed in Mexico (Mills et al., 2010). Therefore, large-scale nationwide epidemiological and epizootiological surveys should be conducted to verify whether human infections occur in Mexico.

In conclusion, we identified three hantaviruses in Mexico: the provisional names are MTN, CAR, and HUI viruses from *P. beatae*, *R. sumichrasti*, and *R. megalotis*, respectively. These viruses are more related to hantaviruses associated with Neotominae rodents, such as LSC, RIOS, and ELMC, than those associated with Sigmodontinae rodents, such as ORO virus, which have previously been identified in *O. couesi* in Mexico. The information obtained in this study is important for understanding the evolution of hantaviruses in the Americas.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2011.11.013.

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Infection of Hantaan virus strain AA57 leading to pulmonary disease in laboratory mice

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ABSTRACT

Hantaan virus (HTNV) is a causative agent of hemorrhagic fever with renal syndrome (HFRS). The pathogenesis of HFRS has not been fully elucidated, mainly due to the lack of a suitable animal model. In laboratory mice, HTNV causes encephalitis. However, that symptom is dissimilar to human hantavirus infections. We found that HTNV strain AA57 (isolated from *Apodemus agrarius* in Far East Russia) caused pulmonary disease in 2-week-old ICR mice. The clinical signs of the infected mice were piloerection, trembling, hunching, labored breathing, and body-weight loss. A large volume of pleural effusion was collected from thoracic cavities of the dead mice. Overall, 45% of the mice inoculated with 3000 focus forming units (FFU) of the virus began to show clinical symptoms at 8 days post-inoculation, and 25% of the inoculated mice died within 3 days of onset of the disease. The morbidity and mortality rates of the mice inoculated with 30–30,000 FFU of HTNV strain AA57 were roughly equivalent. The highest rates of virus positivity (11/12) and the highest titers of HTNV strain AA57 were detected in the lungs of the dead mice, while lower detection rates and viral titers were found in the heart, kidneys, spleen, and brain. Interstitial pneumonia, perivascular edema, hemorrhage, inflammatory infiltration and vascular failure were observed in the lungs of the sick mice. Hantaviral antigens were detected in the lung endothelial cells of the sick mice. The symptoms and pathology of this mouse model resemble those of hantavirus pulmonary syndrome (HPS) and, to a certain extent, those of HFRS. This is the first report that, in laboratory mice, the HFRS-related hantavirus causes a HPS-like disease and shares some symptom similarities with HFRS.

1. Introduction

Hantaviruses are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) in humans (Krüger et al., 2001; Schmaljohn and Hjelle, 1997). Rodents and *Soricomorpha* species are the natural reservoirs for these viruses. Hantaviruses, which are classified in the genus *Hantavirus* within the family *Bunyaviridae*, have a genome that is composed of three negative-stranded RNA segments. The small (S), medium (M), and large (L) genome segments encode nucleocapsid protein (N), two glycoproteins (Gn, Gc), and RNA polymerase, respectively (Schmaljohn, 2001). Each rodent-borne hantavirus has a specific host, and the genus *Hantavirus* contains at present more than 50 species (Jonsson et al., 2010; Kang et al., 2010; Nichol et al., 2005). Rodent-borne hantaviruses are divided into three large groups based on the host animal: *Murinae*-borne; *Arvicolinae*-borne; and *Sigmodontinae*- or *Neotominae*-borne. Many rodent-borne hantaviruses are known to be pathogenic for humans, although these viruses do not cause any apparent symptoms in their natural hosts. Although many hantaviruses have been identified in *Soricomorpha* species, no human disease has been associated with *Soricomorpha*-borne hantaviruses to date (Song et al., 2007).

About 20,000–50,000 cases of HFRS are reported annually in Asia, Europe, and Russia. Five viruses are recognized as the major causative agents of HFRS, including Hantaan virus (HTNV), Amur virus (AMRV) and Seoul virus (SEOV) in East Asia and Far East Russia, and Puumala virus (PUUV) and Dobrava virus (DOBV) in Europe and European Russia (Garanina et al., 2009; Kariwa et al., 2007; Vapalahti et al., 2003). The hosts for HTNV, AMRV, SEOV, and PUUV are *Apodemus agrarius*, *A. peninsulae*, *Rattus norvegicus*, and *Myodes glareolus*, respectively (Schmaljohn, 2001). Recently, a detailed phylogenetic analysis revealed that DOBV forms distinct evolutionary lineages, i.e., DOBV from *A. flavicollis* (DOBV, DOBV-Af), from *A. agrarius* (Saaremaa, DOBV-Aa), and from *A. ponticus*

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(DOBV-Ap) (Avsic-Zupanc et al., 1992; Klempa et al., 2003, 2005, 2008; Nemirov et al., 1999). Regarding HFRS cases reported from East Eurasia, which includes China, Korea, and Far East Russia (Song et al., 2006; Kariwa et al., 2007; Zhang et al., 2010), HTNV is one of the main causes of the disease.

Humans become infected with hantaviruses by either inhaling rodent excreta or being bitten by infected rodents. The initial symptoms of HFRS appear suddenly and include intense headaches, back and abdominal pains, fever, chills, and nausea. Patients show flushing of the face, inflammation, and redness of the eyes, followed by severe symptoms, such as low blood pressure, acute shock, vascular leakage, and acute kidney failure, which can result in severe fluid overload (Peters et al., 1999; Schmaljohn and Hjelle, 1997). The severity of the disease largely depends on the infecting virus. HTNV and DOBV cause severe symptoms, while SEOV and PUUV infections are less severe (Antoniadis et al., 1987; Schmaljohn and Hjelle, 1997). AMRV appears to cause severe HFRS (Lokugamage et al., 2002, 2004b; Yashina et al., 2000, 2001). However, the pathogenesis of HFRS caused by AMRV has not been fully investigated.

Animal models have been developed to uncover the pathogenicity of hantaviruses. Andes virus (ANDV), which is the causative agent of HPS in South America, generates severe symptoms in Syrian hamsters (Campen et al., 2006; Hooper et al., 2001; Wahl-Jensen et al., 2007). The characteristics of ANDV infections in hamsters include rapidly progressing labored breathing, pulmonary edema, pleural effusion, and hypotension, in close resemblance to HPS in humans. In the case of HFRS-related viruses, mice have been used to examine the pathogenesis of HTNV (Ebihara et al., 2000; Kurata et al., 1983; Tamura et al., 1989; Wichmann et al., 2002). However, HTNVs cause neurovirulence in mice, unlike the symptoms associated with hantavirus infections in humans. These results emphasize that the animal models used to analyze the pathogenesis of HTNV need to be improved. Interestingly, cynomolgus macaques infected with PUUV show symptoms similar to those of HFRS (Klingström et al., 2002; Sironen et al., 2008). However, the management of infected nonhuman primates requires a larger biological containment facility and entails higher costs and labor inputs. Thus, it is desirable to establish a suitable and reasonable model with small laboratory animals to analyze the pathogenesis of HFRS.

HTNV strain AA57 (AA57) was isolated from *A. agrarius* in the Khabarovsk region of Russia. The DNA sequences of strain AA57 and of hantaviruses from HFRS patients in the Khabarovsk region were rather similar (nucleotide: 96–99%, amino acid: 98–100%; Kariwa et al., unpublished data).

In the present study, we report on the severe outcome of an infection with strain AA57 in 2-week-old ICR mice. The AA57-infected mice had severe acute pulmonary symptoms, and some of the mice died within 3 days of disease onset. We observed the clinical symptoms of the AA57-infected mice. We analyzed the distribution of the virus in various organs and the associated histopathologic changes, to determine the potential of this model to enhance our understanding of the pathogenesis of hantavirus infections in humans.

2. Materials and methods

2.1. Cells and culture medium

Vero E6 cells (ATCC No. CRL-1586; American Type Culture Collection, Manassas, VA) were maintained in Minimum Essential Medium with Eagle's salts (MEM; Invitrogen, Carlsbad, CA) that was supplemented with 10% fetal bovine serum (FBS; MP Biochemicals, Aurora, OH), 2 mM L-glutamine (Kanto Chemical, Tokyo, Japan), 100 IU/mL penicillin G (Meiji Seika, Tokyo, Japan), and 100 μg/mL streptomycin (Meiji Seika).

2.2. Hantaviruses

HTNV strain AA57 (Genebank ID: S segment; AB620031, M segment; AB620032, L segment; AB620033) and HTNV strain 76-118 cl-1 (cl-1, Genebank ID: S segment; D25530, M segment; D25529, L segment; D25528, Tamura et al., 1989) were used in this study. Virus was inoculated onto a monolayer of Vero E6 cells. The supernatant of the infected cell culture was collected on Day 5, and stored at −80 °C as a working stock. Each virus was passaged twice in Vero E6 cells.

2.2.1. Inoculation of viruses into mice

Two-week-old ICR mice (Japan SLC, Shizuoka, Japan) were used in this study. Ten or twenty mice per group were injected subcutaneously with 0.3 mL of strain AA57 (3–30,000 focus-forming units [FFU]) in MEM. MEM or 30,000 FFU of strain cl-1 were inoculated similarly into 7 or 9 mice per group. After viral challenge, the mice were observed and body weights were measured daily. A reduction in body weight, compared to the previous day, was regarded as onset of the disease. The lungs, kidneys, spleen, liver, heart, brain, and pleural effusion of each dead mouse were collected and stored at −80 °C. Some organs of the dead mice were fixed in 10% neutral-buffered formalin. At 21 days post-inoculation (d.p.i.), all the surviving mice were killed by cervical dislocation under anesthesia, and the organs, blood clots, and sera of each mouse were collected and stored at −80 °C. All animal experiments were performed in a biosafety level 3 animal facility, in accordance with the Guidelines for Animal Experimentation of the School of Veterinary Medicine, Hokkaido University.

2.2.2. Passive immunization before AA57 inoculation into mice

Mice were intraperitoneally injected with 0.3 mL of anti-HTNV strain 76-118 mouse serum or normal mouse serum. One day after injection of the mouse sera, 300 FFU of strain AA57 were inoculated into each group of mice. After viral challenge, the mice were observed and body weights were recorded until 14 d.p.i.

2.3. Virus titration

The method used for hantavirus titration has been described previously (Lokugamage et al., 2004a). The organs of the mice were homogenized in MEM using a cold pestle, mortar, and sea sand. Thereafter, 10% of each homogenate was centrifuged at 2000 × *g* for 5 min, and the supernatant was serially diluted in MEM. Diluted samples (50 μL/well) were inoculated onto monolayers of Vero E6 cells grown in 96-well plates (Nunc, Roskilde, Denmark). After adsorption for 1 h at 37 °C, the inoculum was removed, and 150 μL of MEM that contained 1.5% carboxymethyl cellulose (CMC-MEM) was layered onto the cells, which were then incubated at 37 °C for 5 days in 5% CO_2. After incubation, the monolayers were washed with PBS, fixed with methanol, and air-dried. Foci of hantaviruses were immunostained with an anti-E5/G6 monoclonal antibody (Yoshimatsu et al., 1996) and Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen). Stained foci were counted under a fluorescence microscope.

2.4. Histopathology

Heart, lung, liver, spleen, adrenal gland, kidney, and brain samples were collected from infected animals for histopathology. Tissues were fixed in 10% phosphate-buffered formalin and routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The antigens were retrieved by hydrolytic autoclaving for 10 min at 121 °C in 10 mM sodium citrate–sodium chloride buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in methanol

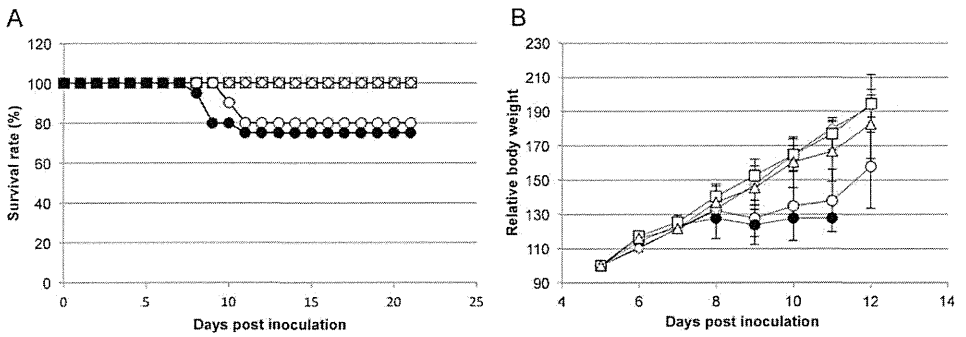

Fig. 1. (A) Survival curve of hantavirus-infected mice (ICR; age, 2 weeks). Mice were inoculated subcutaneously with Hantaan virus strain AA57 (30 or 3000 FFU), Hantaan virus strain 76-118 cl-1 (30,000 FFU) or MEM. Survival rate is expressed as the percentage. Closed circles represent AA57-inoculated mice (3000 FFU, $n=20$); open circles represent AA57-inoculated mice (30 FFU, $n=10$); diamonds represent 76–118 cl-1-inoculated mice ($n=9$); and squares represent MEM-inoculated mice ($n=7$). (B) Body-weight changes of hantavirus-infected mice (ICR; age, 2 weeks). Mice were inoculated subcutaneously with Hantaan virus strain AA57 (3000 FFU), Hantaan virus strain 76-118 cl-1 (30,000 FFU) or MEM. Relative body weight is expressed as the percentage of body weight at 5 days post-inoculation. Closed circles represent AA57-inoculated mice that died ($n=5$); open circles represent AA57-inoculated mice that recovered ($n=4$); triangles represent AA57-inoculated mice that showed no symptoms ($n=11$); diamonds represent 76-118 cl-1-inoculated mice ($n=9$); and squares represent MEM-inoculated mice ($n=7$). The error bars represent standard deviations.

for 30 min. The first antibody added was the anti-E5/G6 monoclonal antibody (1 μg/mL; incubated overnight at 4 °C). The VECTOR M.O.M. Immunodetection Kit (Vector Laboratories, Burlingame, CA) was used to visualize the HTNV antigens in the sections.

3. Results

3.1. HTNV strain AA57 is pathogenic for 2-week-old ICR mice

We had experienced that younger mice produce higher level of antibodies to hantavirus than adult mice. During course of anti AA57 immune serum preparation, we injected strain AA57 to 2-week-old ICR mice and found some of the mice died. In this study, we observed symptoms of the disease and analyzed virus loads as well as pathology in AA57 infected mice.

Two-week-old ICR mice were inoculated subcutaneously with a series of 10-fold dilutions (3–30,000 FFU) of HTNV strain AA57. Ten to twenty mice were used for each dilution of strain AA57, while nine mice were infected with 30,000 FFU of strain cl-1. Similarly, MEM was injected into seven mice as a control. Mice inoculated with strain AA57 began to show clinical symptoms from 7 d.p.i., including piloerection, trembling, hunching, labored breathing, and body-weight loss (Table 1). Some mice inoculated with 30–30,000 FFU of strain AA57 began to die from 7 to 11 d.p.i. (Table 1 and Fig. 1A). Nine of the twenty mice inoculated with 3000 FFU of strain AA57 showed reductions in body weight around 9 d.p.i. (Table 2 and Fig. 1B), and five mice died. Almost all the mouse deaths occurred within 1 day of symptom onset (Table 2). Four of nine sick mice survived and showed an increase in body weight within 1.5 ± 1.59 days of onset (Table 2 and Fig. 1B). Eleven of the twenty mice inoculated with 3000 FFU of strain AA57 showed no symptoms or had body-weight changes that were similar to those of the MEM-injected mice (Table 2 and Fig. 1B). The morbidity and mortality rates of the AA57-inoculated mice (3000 FFU) were 45% (9/20) and 25% (4/20), respectively (Table 2). The morbidity and mortality rates of the mice inoculated with 30–30,000 FFU of strain AA57 showed no significant differences (Table 2). In contrast, none of the mice inoculated with strain cl-1 showed any clinical symptoms, body-weight loss or deaths (Fig. 1A and B).

From the mice that died as a result of inoculation with AA57, 0.1–0.7 mL (average, 0.35 mL) of pleural effusion were collected from the thoracic cavity (Fig. 2). The color of the pleural effusions ranged from yellow to pink. These pleural effusions accounted for, on average, 2.8% of the total mouse body weight. The examinations of the clinical signs and at necropsy indicate that the symptoms seen in AA57-inoculated mice are similar to those see in HPS.

3.2. Viral loads in the organs of mice that died following inoculation with AA57

To determine the distribution of HTNV strain AA57 in the mice that died as a result of viral infection, the organs of these mice were subjected to virus titration (Table 3). Infectious viruses were detected in the lungs, heart, kidneys, spleen, and brain. The highest rates of virus positivity (11/12) and the highest titers of infectious virus were detected in the lungs (Table 3). The rates of virus positivity for the heart, kidneys, spleen, and brain were 4/12, 1/12, 5/12, and 3/10, respectively. The rates of virus positivity and the virus titers in these organs were lower than those in the lungs (Table 3). No virus was detected in any of the liver or pleural effusion samples (Table 3).

3.3. Histopathology

To investigate the pathogenesis of strain AA57, the HTNV AA57-infected mice were subjected to histopathologic analysis. Eight of the ten sick mice had clear histopathologic changes in their lungs at 7–11 d.p.i. (Table 4). The affected mice were shown mild perivascular edema with slight mononuclear cell infiltration around the vessels in the lungs (Fig. 3A–C). In the vessels, degenerated endothelials and adhesion neutrophils were observed and were related virus antigens (Fig. 3D and E). The slight interstitial

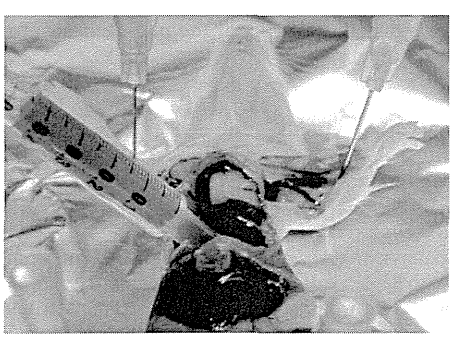

Fig. 2. Collection of the pleural effusions from an AA57-inoculated mouse that died (mouse no. AA57-96).

Table 1
Clinical signs of AA57-infected mice resulting in death.

Mice ID No.	Sex	Titer of inoculated virus (FFU[a])	Onset (d.p.i.)	Death (d.p.i.)	Survival date from onset	Clinical signs	Amount of pleural effusion (mL)
AA57-50	F	30	10	11	1	Body weight loss, labor breathing	0.4
AA57-51	M	30	10	10	0	Found dead	0.7
AA57-60	M	300	10	10	0	Body weight loss, labor breathing	0.4
AA57-64	F	300	10	10	0	Found dead	0.4
AA57-20	M	3000	9	11	3	Body weight loss, labor breathing	0.2
AA57-24	F	3000	9	9	0	Found dead	0.2
AA57-25	M	3000	8	8	0	Found dead	0.4
AA57-66	M	3000	9	9	0	Body weight loss, labor breathing	0.4
AA57-69	F	3000	9	9	0	Found dead	0.7
AA57-96	F	30,000	8	9	1	Body weight loss	0.3
AA57-98	M	30,000	9	9	0	Body weight loss, labor breathing	0.1
AA57-101	M	30,000	7	7	0	Body weight loss, labor breathing	0.2

[a] Focus forming units.

Table 2
Morbidity and mortality in AA57 infected mice.

	Infection dose (FFU[a])				
	3	30	300	3000	30,000
Morbidity	10% (1/10)	50% (5/10)	50% (5/10)	45% (9/20)	70% (7/10)
Average time of onset (d.p.i.)	11	10	10	9.11 ± 0.6[b]	8.14 ± 0.64
Mortality	0% (0/10)	20% (2/10)	20% (2/10)	25% (5/20)	30% (3/10)
No. of recovered animals	1	3	3	4	4
Disease time post onset (day)	1	2.67 ± 2.87	1	1.5 ± 1.59	1.25 ± 0.8
No. of dead animals	0	2	2	5	3
Survival time post (day)	–	0.5	0.5	0.4 ± 0.59	0.3 ± 1.43

[a] Focus forming units.
[b] 95% confidence interval.

pneumonitis with cellular infiltration was also seen in the affected mice (Fig. 3F). These affected mice showed evidence of lymphoid cell depletion in the spleen (data not shown). However, the hantavirus antigen was not detected in the spleen (Table 4). Although infiltration of mononuclear cells and hantavirus antigens were observed in the liver (Table 4), no infectious virus was detected in the liver (Table 3). These results indicate that AA57 infection does not affect liver function.

3.4. Protection against pulmonary disease by the administration of anti-HTNV immune serum

To confirm that, specifically, strain AA57 causes the disease seen in inoculated mice, we administrated intraperitoneally anti-HTNV strain 76-118 immune serum (cross-neutralized to AA57) to mice 1 day before inoculation of the same mice with strain AA57. The immune serum-administered mice were completely protected

Table 3
Virus titers in dead mice.

Mice ID No.	Sex	Titers of inoculated virus (FFU[a])	Virus titers of organs (FFU/g)						
			Lung	Heart	Kidney	Spleen	Brain	Liver	PE[b]
AA57-50	F	30	37,333	54,000	466	–	333	–	–[d]
AA57-51	M	30	1733	–	–	600	–	–	–
AA57-60	M	300	200	–	–	–	N.D.[c]	–	–
AA57-64	F	300	8000	200	–	–	N.D.	–	–
AA57-20	M	3000	400	–	–	–	267	–	–
AA57-24	F	3000	667	–	–	667	2400	–	–
AA57-25	M	3000	5600	–	–	533	–	–	–
AA57-66	M	3000	1200	533	–	1533	–	–	–
AA57-69	F	3000	16,000	–	–	1467	–	–	–
AA57-96	F	30,000	–	–	–	–	–	–	–
AA57-98	M	30,000	4667	867	–	–	–	–	–
AA57-101	M	30,000	400	–	–	–	–	–	–
Positive rate		11/12	4/12	1/12	5/12	3/10	0/12	0/12	

[a] Focus forming units.
[b] Pleural effusion.
[c] Not done.
[d] Virus titer is under detectable level.

Table 4
Histopathological analysis in AA57-infected mice.[a]

ID	Date of onset (d.p.i.)	Collection date (d.p.i.)	Clinical signs	Histopathological changes and virus antigen detection						
				PE[d]	Lung	Heart	Liver	Kidney	Spleen	Brain
AA57-96	8	9[b]	Body weight loss, labor breathing	+ (400 μl)	−/−[e]	−/−	+/+	−/−	+/−	−/−
AA57-98	8	9[b]	Body weight loss	+ (300 μl)	+/−	N.D.[f]	N.D.	−/−	N.D.	N.D.
AA57-101	7	7[b]	Body weight loss, labor breathing	+ (400 μl)	+/−	−/−	+/+	−/−	+/−	−/−
AA57-88	8	8	Body weight loss, labor breathing	−	+/−	−/−	+/+	−/−	+/−	−/−
AA57-89	8	8	Body weight loss, labor breathing	+ (300 μl)	+/−	−/−	−/−	−/−	+/−	−/−
AA57-90	8	8	Body weight loss, labor breathing	−	+/+	−/−	+/+	−/−	+/−	−/−
AA57-93	8	8	Body weight loss, labor breathing	−	+/+	−/−	+/+	−/−	−/−	−/−
AA57-94	8	8	Body weight loss, labor breathing	−	+/+	N.D.	+/+	−/−	N.D.	−/+
AA57-95	8	8	Body weight loss, labor breathing	−	+/−	N.D.	+/+	−/−	+/−	−/−
AA57-91	10	11[c]	Body weight loss	−	−/−	−/−	−/−	−/−	+/−	−/−
AA57-92		11	No clinical sign	−	−/−	−/−	−/−	−/−	+/−	−/−

[a] AA57 was inoculated to each mice 30,000 focus forming units subcutaneously.
[b] Collected from dead mice.
[c] Collected from a recovered mouse.
[d] Pleural effusion.
[e] Histopathological change/virus antigen.
[f] Not done.

against disease caused by strain AA57 and showed no body-weight loss, clinical symptoms, or mortality (data not shown).

4. Discussion

There have been many attempts to develop an animal model that can be used to analyze the pathogenesis of hantaviruses. However, these models have shown only limited suitability for analyzing hantavirus infections. For example, ANDV and Maporal virus cause HPS-like symptoms in Syrian hamsters (Campen et al., 2006; Hooper et al., 2001; Milazzo et al., 2002; Wahl-Jensen et al., 2007). In contrast, some strains of HTNV cause neurovirulence in mice, which is different to the symptoms of human HFRS (Ebihara et al., 2000; Kurata et al., 1983; Tamura et al., 1989; Wichmann et al., 2002). Therefore, there is an urgent need to develop an efficient animal model that will facilitate studies of the pathogenesis of HFRS-related hantaviruses.

We injected strain AA57 to 2-week-old ICR mice to obtain the immune serum and found that strain AA57 causes a severe pulmonary disease. In the present study, we examine the virus loads in organs and the pathology in AA57-infected mice. The AA57-infected mice showed pulmonary symptoms and some of the sick mice died within 1 day of disease onset. This indicates that strain AA57 is pathogenic for 2-week-old ICR mice. In contrast, mice inoculated with HTNV strain 76-118 cl-1 did not show clinical signs.

HTNV is known to cause HFRS, which is characterized by fever, internal hemorrhage, acute shock, vascular leakage, and kidney failure (Peters et al., 1999; Schmaljohn and Hjelle, 1997). However, pulmonary involvement is observed in some HFRS cases attributed to SEOV and PUUV (Caramello et al., 2002; Kikuchi et al., 1982; Linderholm et al., 1992; Rasmuson et al., 2011). In addition, pulmonary edema has been observed in HFRS cases in China, where HTNV, SEOV, and AMRV are prevalent (Hjelle et al., 1995; Zhao et al., 2009). Thus, it is possible that HTNV causes the pulmonary manifestations seen in infected humans. In the present study, large volumes of pleural effusion were collected from the AA57-inoculated mice that died as a result of the infection; this is similar to the pulmonary manifestations of HFRS (described above) and HPS patients. In addition, the pulmonary pathogenesis of AA57 in mice resembled that of ANDV-inoculated hamsters, which could be the models of human HPS (Hooper et al., 2001). The highest titers of infectious virus were detected in the lungs of the AA57-infected mice that died, and hantavirus antigens were detected in the lungs of sick mice by immunohistochemistry. Previous reports have described the detection of hantavirus antigens in the lung endothelial cells of PUUV-infected HFRS cases, HPS patients, and ANDV-infected hamsters (Hooper et al., 2001; Rasmuson et al., 2011; Zaki et al., 1995).

In previous studies, HTNV-inoculated adult or suckling mice developed neurologic diseases, and infectious viruses were detected mainly in the brain, lungs, spleen, and kidneys (Ebihara et al., 2000; Kurata et al., 1983; Tamura et al., 1989; Wichmann et al., 2002). However, the AA57-infected mice showed no neurologic signs, and they had lower rates of virus positivity and lower titers of infectious virus in the brain, heart, kidney, spleen, and liver. These results suggest that AA57 mainly replicates in the lungs, and that the pathologic changes in infected animals occur mainly in this organ. In the histopathologic analyses of AA57-infected mice at 7–11 d.p.i., acute pneumonitis, perivascular edema, hemorrhage, inflammatory infiltrates, and endothelial degeneration were observed in the lungs. Hantavirus antigen was detected in the endothelial cells. These results indicate that increased vascular permeability associated with strain AA57 leads to hemorrhage, accumulation of pleural effusion, and death in the infected mice. Therefore, our data suggest that the AA57-infected ICR mouse model may reflect the pathogenesis of human HPS and, to some extent, that of HFRS. In HFRS cases, renal failure often occurs and hantavirus antigen is detected in the kidneys. In contrast, no histopathologic changes and no infectious virus were found in the kidneys of the AA57-infected mice. These results indicate that AA57 does not replicate well in the kidneys of ICR mice, in contrast with the situation in human HFRS cases. Further investigations are needed to elucidate why strain AA57 does not affect the kidneys of ICR mice.

In the AA57-inoculated ICR mice, although pathologic changes were observed in the lungs, severe destruction of alveolar or endothelial cells was not observed. In addition, hantavirus antigens were scarcely detected in the endothelial cells. The morbidity and mortality rates did not correlate well with the dosages of inoculated virus. In addition, 2-week-old BALB/c inbred mice that were inoculated with AA57 did not show any symptoms (data not shown). These findings suggest that immunologic factors and phenotypes of the infected mice may be important for the development of the disease. Previous reports have described how the neurovirulence of HTNV varies with mouse strain and age (Kariwa et al., 1995; Wichmann et al., 2002). The human HLA haplotype has been associated with the clinical course of acute PUUV infection (Mustonen et al., 1996).

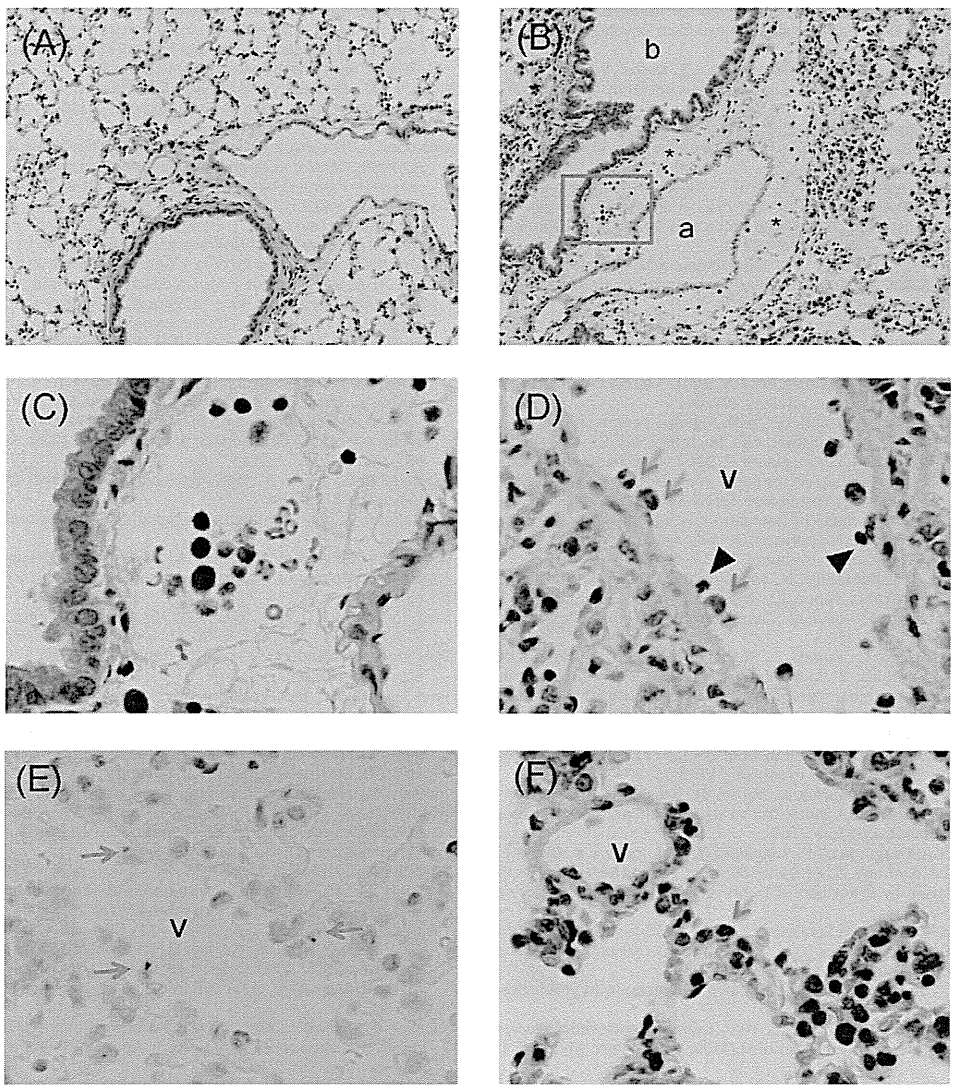

Fig. 3. Histopathological findings in the lungs of 2-week-old ICR mice after hantavirus inoculation. The lungs of mock infected mice (A) and AA57-inoculated mice (B-F) on 8 d.p.i. (A–D and F) Hematoxylin–eosin staining. and (E) immunohistochemistry for hantavirus antigen. a, Artery; b, bronchiole; v, vein. Mild perivascular edema with slight cell infiltration (asterisks) was seen around middle size artery (B). Slight mononuclear cell infiltrations, small hemorrhage, and precipitation of fibrin were found in the edema (C). Degenerated endothelial cells (arrowhead) and adhesion of neutrophils (red arrow) were observed in the middle size vein (D). Virus antigen positive cells were seen in the lesion (E). Slight neutrophils and mononuclear cells infiltration (red arrow) were presented in the alveolar wall (F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Mice inoculated with strain cl-1 showed no symptoms, although strain AA57 and strain cl-1 are genetically and serologically similar (amino acid differences: nucleocapsid, 1.2%; glycoproteins, 2.1%; and RNA polymerase, 1.4%; Kariwa et al., unpublished data). This suggests that small genetic differences between strain AA57 and cl-1 (e.g., those that affect the functions of viral proteins in infected mice) confer differences in HTNV pathogenicity for mice.

In summary, we report on an animal model of old-world hantavirus infection that leads to pulmonary disease. The symptoms observed in this murine model resemble those of HPS and, in certain aspects, those of HFRS. As an experimental animal, mice have several advantages for studies of virus-host interactions. This model may be a useful model for analyzing the pathogenesis of HFRS and hantavirus infections.

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NOTE *Virology*

Application of Truncated Nucleocapsid Protein (N) for Serotyping ELISA of Murinae-Associated Hantavirus Infection in Rats

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ABSTRACT. Truncated recombinant nucleocapsid proteins (trNs) that lack N-terminally located cross-reactive epitopes of four Murinae rodent-associated hantaviruses, Seoul virus (SEOV), Thailand virus, Hantaan virus (HTNV) and Dobrava-Belgrade virus, were produced by using a baculovirus expression system. ELISA with the trNs as antigens enabled serotyping of immune sera from rats experimentally inoculated with the corresponding hantaviruses with cut-off OD values of 60% of those of whole N of HTNV. The trN-based ELISA could serotype 12 out of 13 sera obtained from wild rodents (*Rattus norvegicus*) naturally infected with SEOV using the 60% cut-off value. These results indicate that screening with whole N followed by serotyping with trNs using a cut-off OD value of 60% of that of whole N is a useful method for serological surveillance of Murinae-associated hantavirus infection among rodents.

KEY WORDS: hantavirus, serotyping ELISA.

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Hantaviruses belong to the genus *Hantavirus* in the family *Bunyaviridae* [24]. Hantaviruses are negative-stranded RNA viruses with three segmented RNAs designated small (S), medium (M) and large (L); they encode a nucleocapsid protein (N), envelope glycoproteins (Gn and Gc) and an RNA-dependent RNA polymerase (L protein), respectively [5, 23].

So far, 23 virus species have been registered within the *Hantavirus* genus [22]. Various species of small mammals, mainly rodents and soricomorphs, act as natural reservoirs, as they are infected persistently without any sign of disease [8, 14, 22]. It is generally believed that hantaviruses coevolved with their reservoir animals [7, 19]. As a consequence, hantavirus species have their own predominant reservoirs.

The members of the *Hantavirus* genus contain causative agents of two rodent-borne febrile illness in humans, hemorrhagic fever with renal syndrome (HFRS) in the Old World and hantavirus pulmonary syndrome (HPS) in the New World [22]. Among the Old World hantaviruses, Puumala virus (PUUV), Seoul virus (SEOV), Thailand virus (THAIV), Hantaan virus (HTNV) and Dobrava-Belgrade virus (DOBV) are considered to be causative agents of HFRS [22]. The predominant reservoir rodent species of PUUV is *Myodes glareolus*, which is classified into subfamily Arvicolinae. The predominant reservoir rodent species of SEOV, THAIV, HTNV and DOBV are *Rattus norvegicus*, *Bandicota indica*, *Apodemus agrarius* and *A. flavicollis*, respectively [13, 18, 20], and they are all classified into subfamily Murinae. Despite the close associations between rodent hosts and hantaviruses, exceptional host switching and spillover cases have been reported [26, 27]. Recent studies have shown that *R. tanezumi* in Indonesia and *R. rattus* in Cambodia possessed THAIV-like hantaviruses [20, 21]. Furthermore, prevalence of a novel HTNV was reported among laboratory rats (*Rattus norvegicus*) in China [30]. In our previous study, Da Bie Shan virus, an HTNV-like hantavirus, was detected in *Niviventer confucianus*, a sister genus of *Rattus* [26]. These epizootiologic findings suggest that *Rattus* species rodents act as reservoir animals of various hantaviruses. Therefore, it is important to serotype infected hantaviruses among wild rats to understand the ecology of hantavirus in nature.

Since SEOV, THAIV, HTNV and DOBV possess strong antigenic cross-reactivity in N [3, 4, 28], a neutralization test is the only serological method to define the serotypes [2, 12]. We have developed simple and rapid diagnosis for serotyping ELISA of SEOV, THAIV, HTNV and DOBV infection by using a truncated N that lacks cross-reactive epitopes within the 1–49 N-terminal aa of the N (trN) as an antigen for human sera [1, 15, 29]. In this study, we examined the applicability of the trNs for serotyping in *Rattus* sera by using immune sera from experimentally inoculated rats and naturally infected Norway rats.

SEOV strain SR-11 [9], THAIV strain thai749 [6], HTNV strain 76–118 [11] and DOBV strain Saaremaa [16] were used as representative strains of SEOV, THAIV, HTNV and DOBV serotypes, respectively. Viruses were prepared by the method previously described [1, 15]. Six-week-old WKAH/hkm female rats (SLC, Hamamatsu, Japan) were inoculated intraperitoneally with SEOV (6×10^4 FFU/animal), THAIV (4×10^2 FFU/animal), HTNV (3×10^4 FFU/animal) and DOBV (2×10^4 FFU/animal). Two

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rats were inoculated with each virus. Serum specimens were collected from the tail vein at the time of inoculation (day 0) and at 3, 6, 9, 13, 16, 19, 23, 27, 34, 40 and 49 days post inoculation (dpi) from the same rats. Animal experiments were performed after obtaining permission from the Institutional Animal Care and Use Committee of Hokkaido University. Experiments involving virus infections were performed in a BSL-3 facility.

Serotyping ELISA for IgG antibody was performed essentially by the same procedure as that reported previously [1, 10, 15, 17]. Briefly, truncated N, which lacked 49 N-terminal aa of the N (trN) of SEOV, THAIV, HTNV and DOBV and the whole N of HTNV (whole N) were expressed by a recombinant baculovirus system and used as antigens for ELISA. Serotyping ELISA for IgM was performed as described previously [10] by using polyclonal goat anti-rat IgM (KPL) as a capture antibody. TrN of SEOV, THAIV and HTNV and whole N of HTNV were used as antigens. Serum specimens were examined at 1:400 dilution.

Changes in ELISA OD values of IgG of rats after inoculation are shown in Fig. 1. The ELISA OD values for whole N of HTNV began to appear from 6 to 13 dpi in all rats inoculated with homologous and heterologous viruses and increased to a plateau level until 49 dpi. The higher cross-reactivity of whole N to immune sera from heterologous virus-infected rats indicated the applicability of the whole N of HTNV as a screening antigen to detect a Murinae-derived hantavirus antibody. In all of the eight rats, ELISA OD values of immune sera for homologous trNs were higher than those for heterologous trNs on and after 16 dpi. The OD values for heterologous trNs were <60% of those for whole Ns on or after 16 dpi. However, the type of infected virus could not be differentiated in certain periods, especially in the acute phase, according to our criteria.

The trN-based serotyping ELISA was applied to sera from Norway rats infected naturally with SEOV. A total of 13 sera were obtained from *Rattus norvegicus* captured in Hai Phong Port, Vietnam, and SEOV infection was confirmed by detecting the SEOV genome and indirect immunofluorescent assay (IFA) antibody by methods previously described [25, 27]. The rates of OD values of trNs to those of whole N of HTNV in sera were examined at serum dilutions of 1:200, 400, and 800. As shown in Fig. 2, 4 sera (#1 to #4), which possessed IFA antibody titers of 1:800, could be serotyped at a serum dilution of 1:200 according to the cut-off OD value of 60% of the OD value of whole N. The rest of the serum specimens, except serum #10, were serotyped at 1:400 or 1:800 dilutions, as higher cross-reactivity was obtained at 1:200 dilution. Particularly, serum specimens with IFA antibody titers of more than 1:6,400 required higher dilution (1:800) to be serotyped. The higher cross-reactivity might be due to the presence of high avidity antibody to common epitopes on N produced in persistently infected rats. Nevertheless, our results indicated that the trN-based ELISA was applicable for serotyping of sera from naturally infected Norway rats. Further studies with more rodent sera are required to determine reasonable serum dilutions and cut-off values for serotyping.

In our previous seroepidemiological study in Vietnam, 3 of 12 hantavirus antibody-positive human sera showed very low OD values for trNs of SEOV, THAIV and HTNV, although they reacted strongly to whole N [25]. These unserotyped cases suggested the possibility of the existence of a novel species of hantavirus. In the present study, the OD values of the trN antigens for most of the homologous sera were more than 60% of those for whole N antigen, while the OD values of trN antigens for heterologous sera were less than 60% (Fig. 1). Therefore, novel hantavirus infection would be defined if the OD values of a serum specimen for all the trN antigens are less than 60% of those of whole N antigens. Further studies with more rodent sera serotyped by a neutralization test are needed to determine more reasonable cut-off values for distinguishing a previously known hantavirus infection from a novel hantavirus infection.

We also attempted to apply the trNs for IgM serotyping ELISA. As shown in Fig. 3, ELISA IgM OD values for whole N were transiently increased in SEOV-infected rats, S1 (6 dpi) and S2 (9 and 13 dpi). However, very low reactivities were observed with trNs of SEOV, THAIV and HTNV. The lack of success using trNs for IgM serotyping ELISA is probably due to the general characteristics of IgM antibody, which is broadly reactive with low affinity.

In summary, screening with whole N followed by the trN-based serotyping ELISA is useful in epizootiological surveillance of hantavirus infection among rodents for detecting host switching and spillover as well as antigenically distinct hantavirus infections.

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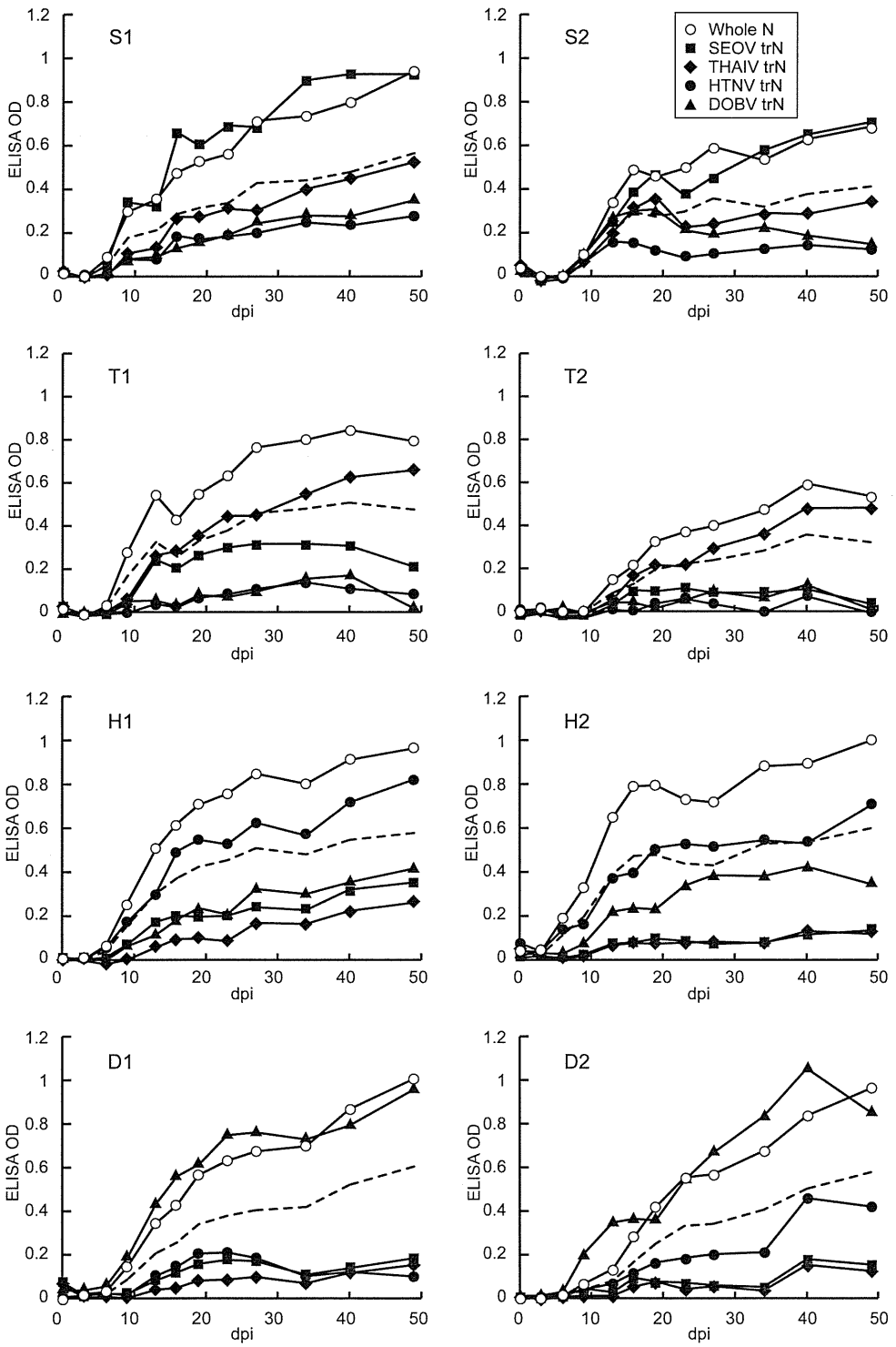

Fig. 1. Changes in ELISA OD values of IgG of rats inoculated with SEOV (S1 and S2), THAIV (T1 and T2), HTNV (H1 and H2) and DOBV (D1 and D2). Open circles show OD values for whole N of HTNV, and black symbols show OD values for trN of SEOV (square), THAIV (diamond), HTNV (circle) and DOBV (triangle). Broken lines show the 60% levels of OD values for whole N.

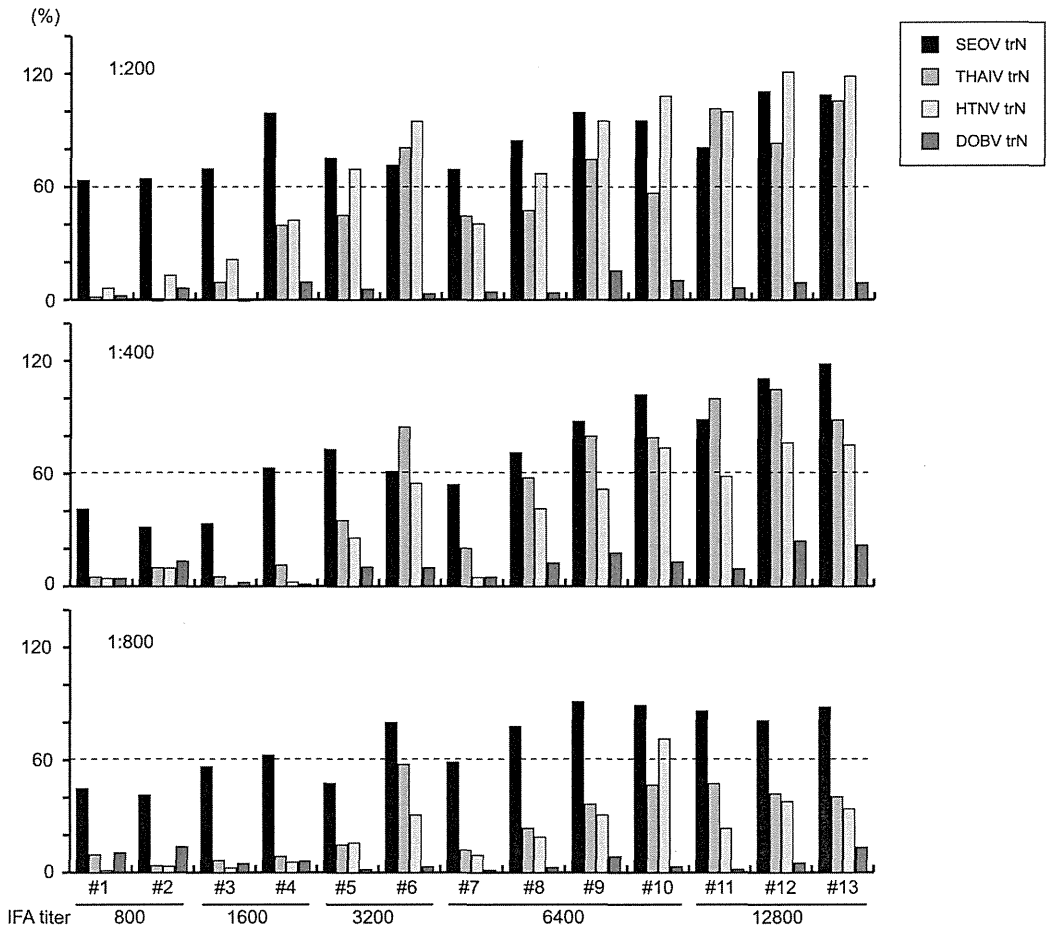

Fig. 2. Rates of OD values of trNs to those of whole N of HTNV. The thirteen sera from rats infected with SEOV were applied to the serotyping ELISA at dilutions of 1:200 (upper panel), 1:400 (middle panel) and 1:800 (lower panel).

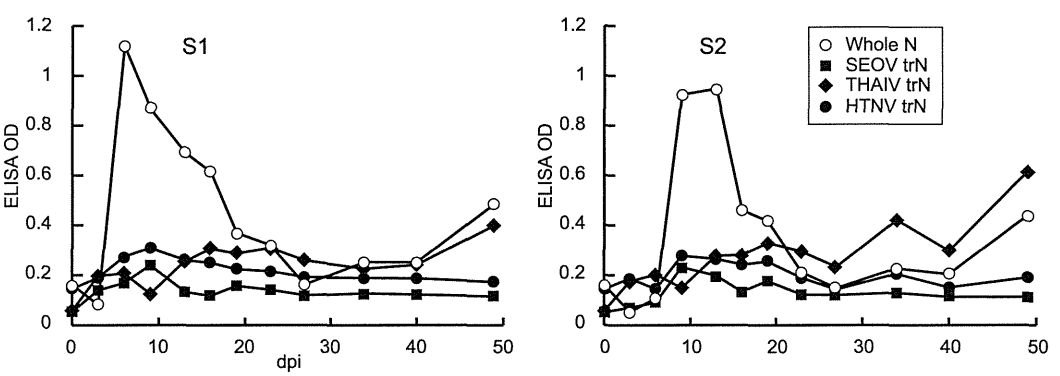

Fig. 3. Changes in ELISA OD values of IgM of rats inoculated with SEOV (S1 and S2). Open circles show OD values for whole N of HTNV, and black symbols show OD values for trN of SEOV (square), THAIV (diamond) and HTNV (circle).

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