

## METHODS

**Cells.** BHK-21 cells were grown at 37 °C in minimum essential medium supplemented with 8% (v/v) FCS and L-glutamine. Human embryonic kidney 293T cells were cultured at 37 °C in Dulbecco's modified Eagle's medium, containing 10% (v/v) FCS, L-glutamine and penicillin/streptomycin.

**Antibodies.** For the detection of TBEV prM and E proteins by ELISA, immunoprecipitation and immunofluorescence experiments, mouse anti-E mAbs 1H4 and 4H8, prepared in our laboratory, were used (Komoro *et al.*, 2000). Rabbit polyclonal anti-prM and anti-E antibodies were prepared by immunization with recombinant prM and E proteins, as described previously (Yoshii *et al.*, 2004). Mouse anti-E mAb 10B4 was kindly provided by Dr Konishi (Kobe University Graduate School of Medicine, Kobe, Japan) for the detection of JEV E protein by Western blotting. For the immunofluorescence co-localization studies, anti-calreticulin rabbit polyclonal antiserum (Affinity BioReagents) or anti-giantin rabbit polyclonal antiserum (Covance Research Products) was used. FITC-conjugated anti-mouse IgG antibodies and Texas Red-conjugated anti-rabbit IgG antibodies (Jackson ImmunoResearch) were used as secondary antibodies in the immunofluorescence assays, and alkaline phosphatase-conjugated anti-rabbit and anti-mouse IgG antibodies (Jackson ImmunoResearch) were used in the Western blot analysis.

**Plasmids.** Plasmid pCAG-TBEME, a pCAGGS-based plasmid encoding the TBEV (Oshima 5-10 strain) signal sequence of the prM, and the prM and E genes, was constructed as described previously (Yoshii *et al.*, 2003).

Plasmid pcJEME, a pcDNA3-based plasmid that encodes the JEV (Nakayama strain) signal sequence of prM, and the prM and E genes, was kindly provided by Dr Konishi and by Dr Mason (Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA) (Konishi *et al.*, 1998).

The TBEV infectious cDNA clone used, Oshima-IC, was constructed previously (Hayasaka *et al.*, 2004).

All mutations within the conserved sequence of the prM protein were engineered in the pCAG-TBEME, pcJEME and Oshima-IC full-length plasmid constructs using standard PCR mutagenesis techniques (Table 2).

**Expression of viral prM and E proteins.** 293T cells were transfected with each plasmid complexed to *TransIT-LT1* reagent (PanVera) in Opti-MEM (Invitrogen), as described previously (Yoshii *et al.*, 2003). After a 24 h incubation, the cells and supernatant were harvested and used for further experiments.

**ELISA.** The E protein of TBEV was detected by ELISA, as described previously (Yoshii *et al.*, 2003). Briefly, transfected cells were lysed with 1% (v/v) Triton X-100 in 10 mM Tris-buffered saline (TBS) and the supernatants were treated with 1% Triton X-100. Triton X-100-solubilized samples were added to mAb 1H4-coated wells of 96-well microtitre ELISA plates, previously blocked with 3% (w/v) BSA. The E protein was detected by incubation with biotinylated mAb 4H8 and HRP-conjugated streptavidin (Sigma). HRP activity was detected by adding *o*-phenylenediamine dihydrochloride (Sigma) in the presence of 0.03% (v/v) H<sub>2</sub>O<sub>2</sub> and absorbance was measured at 450–620 nm.

**Immunoprecipitation.** 293T cells were transfected with pCAG-TBEME containing the various mutations, as described above. After a 24 h incubation, the cells were lysed with Triton X-100 in 10 mM TBS, incubated on ice for 20 min and centrifuged (16 000 g, 20 min). The supernatant, which excluded the nuclear fraction, was pre-cleared on protein G-Sepharose beads (Amersham Pharmacia Biotech) for

2 h at 4 °C. Pre-cleared lysates were combined with protein G-Sepharose beads with mAb 1H4 and precipitated by incubation for 2 h at 4 °C. Immune complexes were pelleted (10 000 g, 10 s) and washed four times with 1% Triton X-100 in 10 mM TBS. Subsequently, the precipitated materials were solubilized and analysed by SDS-PAGE and Western blotting.

**SDS-PAGE and Western blotting.** Protein samples were separated by SDS-PAGE [12% (w/v) acrylamide]. The protein bands were transferred onto PVDF membranes and incubated with 1% (w/v) gelatin in 25 mM TBS containing 0.01% (v/v) Tween 20 (TBST). After washing with TBST, the membranes were reacted with an antibody against the viral protein of TBEV or JEV, followed by alkaline phosphatase-conjugated secondary antibody. Protein bands were visualized using an alkaline phosphatase detection reagent kit (Novagen), according to the manufacturer's protocol.

**Immunofluorescence assay.** 293T cells were grown on eight-well chamber slides (Nalge Nunc International) and transfected with pCAG-TBEME containing the various mutations. After 8 h incubation, cells were rinsed with PBS, fixed with 4% (w/v) paraformaldehyde for 10 min and permeabilized with 0.2% (v/v) Triton X-100 for 4 min at room temperature. After blocking with 2% (w/v) BSA, the cells were incubated for 1 h with mouse mAb 1H4 and antibodies that recognize marker proteins of various cellular organelles or anti-prM antibody. After extensive washing, the cells were incubated with fluorescently labelled conjugated secondary antibodies. The cells were washed three times with PBS, followed by mounting of the coverslips on glass slides. Images were viewed and collected with an Olympus IX70 confocal microscope.

**Electron microscopy.** 293T cells were transfected with pCAG-TBEME containing the various mutations. After a 24 h incubation, the cells were harvested and centrifuged (1000 g, 5 min). The pellets were fixed with 3% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h and rinsed three times with 0.1 M phosphate buffer. After post-fixation in a 1% (w/v) osmium tetroxide solution for 1.5 h, the pellets were dehydrated through a series of graded ethanols and embedded in Epon 812 via QY1 (Nishin EM). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined under a JEM 1210 transmission electron microscope (JEOL) at an acceleration voltage of 80 kV.

**Virus recovery and titration.** Infectious RNA was transcribed from the Oshima-IC plasmid using an mMACHINE SP6 kit (Ambion) and electroporated into BHK-21 cells, as described previously (Hayasaka *et al.*, 2004). At 1–5 days post-electroporation, the secreted virus was recovered and the infectious virus titre was assayed using a focus count assay, as described previously (Takahima *et al.*, 1997). Briefly, monolayers of BHK-21 cells were grown in 96-well plates and inoculated with serial dilutions of the virus. After 38 h incubation, virus foci were visualized by immunofluorescent staining using anti-TBEV E antibodies.

**Passaging experiments.** Passages of the virus on BHK-21 cells were performed by transferring aliquots (200 µl) of cell culture supernatants (at 3 days post-infection) cleared of cell debris and insoluble material by low-speed centrifugation to fresh BHK-21 cells grown in 24-well culture plates.

**Molecular modelling.** The model structure for an asymmetrical trimer of prM–E heterodimers was generated by superimposing the crystal structure of dengue 2 virus (Protein Data Bank code: 3C6E) onto the pseudo-atomic structure determined by cryo-electron microscopy (Protein Data Bank code: 3C6D). This structure was refined by molecular dynamics simulations followed by energy minimizations. First, the system was gradually heated from 50 to

1000 K for 13 ps of simulation and then cooled to 300 K for 10 ps. A harmonic constraint of  $10 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$  was applied on the C $\alpha$  atoms of the protein during the simulation. The time step was set to 1 fs. After molecular dynamics simulations, the energy minimization was conducted without positional constraints using 10 000 steps of steepest descent, followed by conjugate gradient minimization, until the root mean square gradient was  $\leq 0.01 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ . All molecular dynamics and energy minimization calculations were performed using Discovery Studio 2.5 (Accelrys, <http://accelrys.com/products/discovery-studio/>) with a CHARMM force field with a generalized Born implicit solvent model.

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## Isolation and Characterization of Hantaviruses in Far East Russia and Etiology of Hemorrhagic Fever with Renal Syndrome in the Region

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**Abstract.** Hemorrhagic fever with renal syndrome (HFRS) is a serious public health issue in Far East Russia. Two different hantaviruses were isolated from rodents captured in the Khabarovsk region: Amur virus (AMRV; Khekhtsir/AP209/2005 strain from *Apodemus peninsulae*) and Hantaan virus (HTNV; Galkino/AA57/2002 strain from *A. agrarius*). Genetic analysis of the new isolates revealed that the M and L segments were apparently different between AMRV and HTNV, but S segments of the two viruses were closer. The antigenicities of AMRV, HTNV, and Seoul virus (SEOV) were differentiated by cross-neutralization. Serological differential diagnoses of 67 HFRS patients in the Primorsky and Khabarovsk regions of Far East Russia were conducted using a neutralization test. The results revealed that the major cause of HFRS varied with location in Far East Russia: SEOV for Vladivostok city in the Primorsky region, AMRV in rural areas of the Primorsky region, and probably HTNV for the Khabarovsk region.

### INTRODUCTION

Hantaviruses are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) in humans.<sup>1,2</sup> Rodents and *Soricomorpha* species are natural reservoirs of these viruses, and humans acquire infection by inhaling the excreta of infected animals. Hantaviruses are classified in the genus *Hantavirus* within the family *Bunyaviridae*, and they possess a genome composed of three negative-stranded RNA segments. The small (S), medium (M), and large (L) genome segments encode nucleocapsid protein (N), two glycoproteins (Gn and Gc), and RNA polymerase, respectively.<sup>3</sup> Each rodent-borne hantavirus has its own host, and more than 40 species of hantaviruses have been identified.<sup>4,5</sup> Some rodent-borne hantaviruses cause HFRS or HPS and are considered to be important zoonotic agents in various countries around the world.

About 20,000–50,000 cases of HFRS are reported annually worldwide, and large proportions of the infections are from the East Eurasian continent, including China, Korea, and Far East Russia.<sup>6–8</sup> Hantaan virus (HTNV) and Seoul virus (SEOV) are known to cause severe and mild forms of HFRS in the Eastern Eurasian Continent, respectively.<sup>2,9,10</sup> HTNV is carried by the striped field mouse, *Apodemus agrarius*, which preferentially inhabits grass and rice fields. Thus, humans seem to acquire HTNV infection primarily in fields. However, SEOV is carried by the brown rat, *Rattus norvegicus*, and the black rat, *R. rattus*, which are peridomestic rodents. Thus, humans tend to become infected with SEOV in cities and close to dwellings.<sup>8,11–14</sup> Recently, Amur virus (AMRV; also known as Soochong virus) was identified in the Korean field mouse *A. peninsulae* and in HFRS cases in China, Korea, and Far East Russia.<sup>11–13,15,16</sup> Thus, AMRV should also be con-

sidered as one of the causative agents of HFRS in East Asia and Far East Russia.

Morbidity of HFRS is 1.9 per 100,000 population, and about 100–200 HFRS cases are reported annually in Far East Russia.<sup>17</sup> In addition, seroprevalence in healthy residents in Khabarovsk and Primorsky regions is about 2.4% and 2.7%, respectively.<sup>18,19</sup> Despite the basic epidemiological information, an etiological analysis of HFRS has not been carried out in this region because of the close antigenicities between the viruses. Usual serological methods, such as indirect immunofluorescent antibody assays, cannot differentiate the infections caused by AMRV and HTNV. Additionally, genetic or antigenic information about the viruses circulating in the region remains extremely limited.

In the present study, we successfully isolated HTNV and AMRV from *A. agrarius* and *A. peninsulae*, respectively, in Far East Russia. Furthermore, the genetic and antigenic properties of these isolates were compared with the properties of other hantaviruses. Additionally, a serological differential diagnosis of HFRS patients in the Primorsky and Khabarovsk regions of Far East Russia was performed using a neutralization test to distinguish HTNV, AMRV, and SEOV infections.

### MATERIALS AND METHODS

**Rodent survey.** Epizootiological surveys targeting rodents were conducted in Khekhtsir, about 20 km south from Khabarovsk, and Galkino, about 20 km east from Khabarovsk, Russia, in 2002 and 2005, respectively. Animals were captured using live traps set in forests and fields at these survey locations. Live animals were killed by cardiac puncture under anesthesia by ether inhalation, and serum was separated from the collected blood. From dead animals, blood samples were collected using filter paper. The paper was air-dried and immersed in a 10× volume of phosphate-buffered saline (PBS) at 4°C overnight, and the supernatant was used as 10× diluted serum. The sera were heat-inactivated at 56°C for 30 minutes and then stored at –40°C until use. The lungs were collected from all rodents and stored at –80°C until use.

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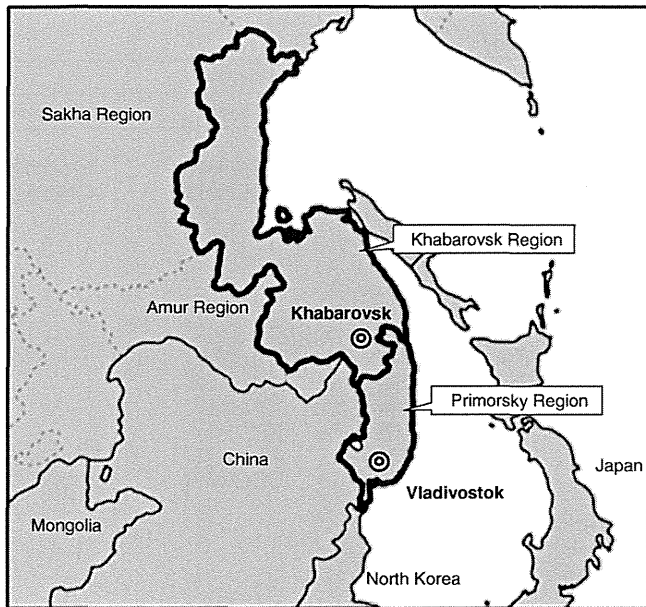


FIGURE 1. Geographical locations of epizootiological survey sites in Far East Russia. Rodents were captured in the Khabarovsk region. Sera of HFRS patients were collected in the Primorsky and Khabarovsk regions.

**Human sera.** Sera from 67 HFRS patients in the Khabarovsk and Primorsky regions, Russia, from 1965 to 2002 (Figure 1) were tested for neutralizing antibodies to hantaviruses. These sera were heat-inactivated and stored at  $-40^{\circ}\text{C}$  until use.

Patient sera were collected with the informed consent of the patients. All experiments using patient sera were approved by the Ethics Committee of Hokkaido University and were performed in a BSL-3 laboratory at the Graduate School of Veterinary Medicine, Hokkaido University.

**Cells and media.** Vero E6 cells (No. CRL-1586; ATCC, Manassas, VA) were maintained in minimum essential medium with Eagle's salts (MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin G, and 100  $\mu\text{g}/\text{mL}$  streptomycin.

**Indirect immunofluorescent antibody assay.** For the detection of antihantavirus antibodies in wild rodents, immunofluorescent antibody assay (IFA) was carried out as described previously.<sup>8</sup> Vero E6 cells were infected separately with HTNV 76-118,<sup>20</sup> Bao14,<sup>21</sup> AMRV H5,<sup>22</sup> SEOV SR-11,<sup>23</sup> or PUUV Sotkamo, and the infected cells were fixed using cold acetone on 24-well slides. Wild rodent sera, including sera from dead animals, diluted 1:16 with PBS were spotted onto the slides and incubated at  $37^{\circ}\text{C}$  for 1 hour. The slides were washed three times with PBS, and then, Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG; Invitrogen) was applied on the slides. After 1 hour of incubation and washing, the cells were observed under a fluorescence microscope. Scattered and fine granular fluorescence in the cytoplasm of Vero E6 cells was considered to indicate a positive reaction. Positive samples were further tested to determine IFA titer, which was the reciprocal of the highest serum dilution giving the positive reaction.

For the detection of hantaviral antigens in Vero E6 cells, the inoculated cells on 24-well slides were visualized using a monoclonal antibody E5/G6 to hantavirus N<sup>24</sup> and Alexa Fluor 488 goat anti-mouse IgG.

**Reverse transcription polymerase chain reaction.** Total RNA from lung tissue of rodents or Vero E6 cells was extracted using ISOGEN (Nippon Gene Co.). Purified total RNA (5  $\mu\text{g}$ ) was reverse-transcribed using Superscript II RNase H reverse transcriptase (Invitrogen) and random primers (Invitrogen) according to the manufacturer's protocol. All segments of hantaviruses were amplified by polymerase chain reaction (PCR) using Platinum Taq DNA polymerase Hi Fidelity (Invitrogen). The thermal conditions for PCR were  $94^{\circ}\text{C}$  for 2 minutes followed by 35 cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $50\text{--}60^{\circ}\text{C}$  (depending on the primer used) for 30 seconds, and  $68^{\circ}\text{C}$  for 2 minutes. To detect hantavirus RNA from wild rodents, primers AMR595SFw ( $5'\text{-agcatgaaggcagaagagat-3}'$ ) and Bao14\_840SRv ( $5'\text{-ctgccgtagtagtcctgt-3}'$ ) were used to detect HTNV, and primers AMR595SFw and AMR1252SRv ( $5'\text{-ctctgtgctagtgctctcaa-3}'$ ) were used to detect AMRV.

**Virus isolation.** The method of hantavirus isolation has been described previously.<sup>25</sup> Lung tissue from both hantavirus antibody- and RNA-positive wild rodents was selected for inoculation in Vero E6 cells. Lungs of *A. agrarius* and *A. peninsulae* were homogenized in MEM using a cold pestle, mortar, and sea sand. Part (10%) of the homogenate was centrifuged ( $2,000 \times g$  for 5 minutes), and each supernatant was inoculated in Vero E6 cells grown in a 25-cm<sup>2</sup> flask by centrifugation ( $670 \times g$ ) for 1 hour at room temperature. After the inoculum was discarded, the cells were cultured at  $37^{\circ}\text{C}$  in a 5% CO<sub>2</sub> incubator and then subcultured after a 14-day interval. At subculture, some cells were collected and spotted on 24-well slides at  $37^{\circ}\text{C}$  for 4 hours in 5% CO<sub>2</sub>. The slides were fixed with cold acetone and used as antigen slides for IFA. The remaining collected cells were examined for hantaviral RNA by reverse transcription (RT)-PCR as described above.

**Sequencing hantavirus genes.** The open reading frames (ORFs) of all segments of newly isolated hantaviruses were amplified using specific primers. PCR products were extracted and purified from agarose gels using the Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI) and sequenced directly using the BigDye Terminator (version 3.1) Cycle Sequencing Kit and an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

**Genetic analysis.** Hantavirus nucleotide and amino acid sequences were compared using Genetyx-mac (version 10.0; Genetyx Corp., Tokyo, Japan) for calculating nucleotide and amino acid identities among hantaviruses. MEGA 4 (available at <http://www.megasoftware.net/mega.html>) was used to generate multiple alignments. A GTR++I model was selected as the best probability model of Bayesian phylogenetic tree of each hantavirus genome segment by MrModeltest software (version 2.3).<sup>26</sup> Phylogenetic trees were drawn using MrBayes (version 3.1.2).<sup>27</sup> Two independent, parallel Markov chain Monte Carlo Metropolis coupling (MCMCMC) analyses, each with four chains (three heated and one cold), were computed for 0.7–1.5 million generations, with tree sampling every 100 generations. A burn-in period of 175,000–375,000 generations was discarded for each run before calculating consensus trees. After two runs of calculation, 10,500–22,500 trees were made, and these trees were used for generating the consensus tree. The sequences of hantaviruses determined in this study were downloaded from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>) and are listed in Table 1.

TABLE 1  
Hantavirus sequences used in this study

Strain	Source	Capture site		Genbank ID		
		Country	Region	S segment	M segment	L segment
Newly isolated virus						
Khekhtsir/AP209/2005	<i>A. peninsulæ</i>	Russia	Khabarovsk	AB620028	AB620029	AB620030
Galkino/AA57/2002	<i>A. agrarius</i>	Russia	Khabarovsk	AB620031	AB620032	AB620033
Amur virus						
Solovey/AP61/1999	<i>A. peninsulæ</i>	Russia	Primorsky	AB071183	—*	—
Solovey/AP63/1999	<i>A. peninsulæ</i>	Russia	Primorsky	AB071184	—	—
AP1371	<i>A. peninsulæ</i>	Russia	Khabarovsk	AF427324	—	—
AP1168	<i>A. peninsulæ</i>	Russia	Khabarovsk	AF427323	—	—
AP708	<i>A. peninsulæ</i>	Russia	Khabarovsk	AF427322	—	—
H5	Human	China	Heilongjiang	AB127996	AB127993	—
B78	Human	China	Shandong	AB127997	AB127994	—
Liu	Human	China	Shandong	AF288649	AF288648	—
JilinAP06	<i>A. peninsulæ</i>	China	Jilin	EF121324	EF371454	—
SC-1	<i>A. peninsulæ</i>	Korea	Gangwon	AY675349	AY675353	DQ056292
SC-2	<i>A. peninsulæ</i>	Korea	Gangwon	AY675350	DQ056293	AY675354
Hantaan virus						
AA1028	<i>A. agrarius</i>	Russia	Khabarovsk	AF427318	—	—
AA1719	<i>A. agrarius</i>	Russia	Khabarovsk	AF427319	—	—
AA2499	<i>A. agrarius</i>	Russia	Khabarovsk	AF427320	—	—
KHB/HFRS/Pat#A2	Human	Russia	Khabarovsk	—	AB086620	—
KHB/HFRS/Pat#A3	Human	Russia	Khabarovsk	—	AB086619	—
KHB/HFRS/Pat#A12	Human	Russia	Khabarovsk	—	AB086621	—
Bao14	<i>A. agrarius</i>	China	Heilongjiang	AB127998	AB127995	—
CGHu1	Human	China	Guizhou	EU092218	EU092222	—
S85-46	<i>A. agrarius</i>	China	Sichuan	AF288659	AF288658	—
76-118	<i>A. agrarius</i>	Korea	Gyong Gi	NC_005218	NC_005219	NC_005222
CUMC-B11	Not known	Korea	—	U37768	U38117	—
CFC94-2	Human	Korea	—	X95007	—	—
Maaji-2	Human	Korea	—	AF321095	—	—
Q32	<i>A. agrarius</i>	China	Guizhou	AB027097	DQ371905	DQ371906
CGAa4MP9	<i>A. agrarius</i>	China	Guizhou	EF990915	EF990929	—
84FLi	<i>A. agrarius</i>	China	Shaanxi	AY017064	AF345636	AF336826
A9	<i>A. agrarius</i>	China	Jiangsu	AF329390	AF035831	AF293665
Z10	Human	China	Zhejiang	NC_006433	NC_006437	NC_006435
Seoul virus						
SR-11	<i>Rattus norvegicus</i>	Japan	Hokkaido	M34881	M34882	—
80-39	<i>R. norvegicus</i>	Korea	Seoul	NC_005236	NC_005237	NC_005238
Puumala virus						
Sotkamo	<i>Myodes glareolus</i>	Finland	—	NC_005224	NC_005223	NC_005225

\* Not registered or not used in this study.

**Immune sera.** Antisera to each hantavirus were obtained from hantavirus-immunized mice. Two-week-old BALB/c mice (SLC, Hamamatsu, Japan) were inoculated subcutaneously (s.c.) with 3,000 focus-forming units (FFUs) of two strains of newly isolated hantaviruses. At 70 days post-inoculation (d.p.i.), mice were killed by cardiac puncture under anesthesia with sevoflurane, and the sera were collected. The sera were heat-inactivated and stored at  $-40^{\circ}\text{C}$  until use.

All animal experiments were performed according to the Guidelines of Animal Experimentation of the School of Veterinary Medicine, Hokkaido University. All animal experiments were carried out in a biosafety level 3 animal facility.

**Focus reduction neutralization test.** The protocol for focus reduction neutralization test (FRNT) has been described previously.<sup>28</sup> Immune mouse sera were used to analyze serological relationships among newly isolated hantaviruses and other hantaviruses. Additionally, 67 human sera from HFRS patients in the Primorsky and Khabarovsk regions were used to identify the serotype of hantaviruses infection in each patient. Serially diluted samples (100  $\mu\text{L}$ ) were mixed with an equal volume of stock viruses (200 FFUs/100  $\mu\text{L}$ ), and these mixtures were incubated at  $37^{\circ}\text{C}$  for 1 hour in 5%  $\text{CO}_2$ . The mixtures (50  $\mu\text{L}$ /well) were inoculated onto

Vero E6 cell monolayers grown in 96-well plates (Nalge Nunc, Roskilde, Denmark). After adsorption for 1 hour at  $37^{\circ}\text{C}$ , the inoculum was removed, and 150  $\mu\text{L}$  MEM with 1.5% carboxymethyl cellulose (CMC-MEM) were layered onto the cells and incubated at  $37^{\circ}\text{C}$  for 4 days in 5%  $\text{CO}_2$ . After incubation, the monolayers were washed with PBS, fixed with methanol, and air-dried. Foci of hantaviruses were immunostained with E5/G6 mAb and Alexa Fluor 488 goat anti-mouse IgG. Stained foci were counted under a fluorescence microscope. The neutralization titer was expressed as a reciprocal of the highest dilution that showed  $\geq 80\%$  inhibition of virus focus formation.

**Statistical analysis.** Statistical analysis for the comparison of the proportions of serotypes among HFRS patients living at different sites was performed using the  $\chi^2$  test. Haberman's residual analysis was conducted to examine which hantavirus was the major cause of HFRS in the Khabarovsk and Primorsky regions.<sup>29</sup>

## RESULTS

**Rodent survey.** We captured 29 *A. peninsulæ* in the forest of Khekhtsir close to Khabarovsk city in 2005 and

TABLE 2  
Detection of antihantavirus antibodies and virus RNA in *A. peninsulae* ( $N = 29$ ) and *A. agrarius* ( $N = 47$ )

Number	IFA antibody titer*				RT-PCR	Virus isolation
	AMRV H5	HTNV 76-118	SEOV SR-11	PUUV Sotkamo		
<i>A. peninsulae</i>						
167	256	512	< 16	< 16	+	N.D.†
<b>172‡</b>	<b>256</b>	<b>256</b>	<b>&lt; 16</b>	<b>&lt; 16</b>	+	-
186	256	512	< 16	< 16	+	N.D.
199	512	128	< 16	< 16	+	N.D.
<b>209</b>	<b>1,024</b>	<b>1,024</b>	<b>128</b>	<b>&lt; 16</b>	+	+
161	512	512	< 16	< 16	-	N.D.
170	512	128	256	< 16	-	N.D.
189	1,024	128	128	< 16	-	N.D.
190	32	16	< 16	< 16	-	N.D.
191	1,024	512	512	< 16	-	N.D.
166	< 16	< 16	< 16	< 16	+	N.D.
Others ( $N = 18$ )	< 16	< 16	< 16	< 16	-	N.D.
<i>A. agrarius</i>						
<b>56</b>	> 2,048	> 2,048	<b>128</b>	< 16	+	+
<b>57</b>	> 2,048	> 2,048	<b>512</b>	< 16	+	+
<b>61</b>	<b>1,024</b>	<b>1,024</b>	<b>256</b>	< 16	+	+
<b>65</b>	> 2,048	> 2,048	> 2,048	< 16	+	+
54	> 2,048	> 2,048	256	< 16	-	N.D.
Others ( $N = 42$ )	< 16	< 16	< 16	< 16	-	N.D.

\* IFA antibody titer was expressed as a reciprocal of the highest dilution that showed specific fluorescence.

† N.D. = not done.

‡ Rodent samples in bold were used for virus isolation.

47 *A. agrarius* in the field of Galkino near Khabarovsk city in 2002 (Table 2). Ten *A. peninsulae* (34.5%) and five *A. agrarius* (10.6%) were seropositive for hantaviruses. Antibody titers against AMRV in *A. peninsulae* ranged from 1:32 to 1:1,024, which was fairly comparable with the titers against HTNV. Antibody titers against SEOV were apparently lower than the titers against AMRV in 8 of 10 seropositive *A. peninsulae*. Antibody titers against HTNV in the five seropositive *A. agrarius* ranged from 1:1,024 to more than 1:2,048, which was equivalent to the titers against AMRV. Four of five seropositive *A. agrarius* had IFA antibody titers against AMRV or HTNV that were more than or equal to fourfold greater than the titers against SEOV. No antibody was detected against Puumala virus (PUUV) in any animal. Lung samples of all 76 rodents were tested for the hantavirus S gene by RT-PCR (Table 2). Six *A. peninsulae* (20.7%) and four *A. agrarius* (8.5%) contained virus RNA.

**Virus isolation.** Virus isolation was carried out using two lung samples of *A. peninsulae* (numbers 172 and 209) and four samples of *A. agrarius* (numbers 56, 57, 61, and 65) that were sero- and RNA-positive. Lung homogenates of each sample were inoculated on Vero E6 cells. At 14 d.p.i., hantavirus RNA was detected from cells inoculated with *A. peninsulae* number 209 and *A. agrarius* numbers 56, 57, 61, and 65. Hantavirus N was detected at 42 d.p.i. (*A. peninsulae* number 209) and 28 d.p.i. (*A. agrarius* numbers 56, 57, 61, and 65). Thus, five strains of hantavirus were isolated from wild rodents (Table 2). Hantavirus isolates from *A. peninsulae* number 209 and *A. agrarius* numbers 56, 57, 61, and 65 were designated Khekhtsir/AP209/2005 (AP209), Galkino/AA56/2002 (AA56), Galkino/AA57/2002 (AA57), Galkino/AA61/2002 (AA61), and Galkino/AA65/2002 (AA65), respectively. AP209 and AA57 were used for additional genetic and antigenic characterization.

**Genetic analysis of hantavirus isolates.** Nucleotide sequences covering the ORF of all segments from AP209 and AA57 were determined. The sequences determined and used in this study are listed in Table 1.

The S segment of AP209 was quite similar to the segments of AMRV Solovey/AP61/1999 and Solovey/AP63/1999 that were detected from *A. peninsulae* in the Primorsky region, Russia (96.0–96.3% nucleotide identity and 99.3–99.8% amino acid identity) (Table 3). The identities in the S segment between AP209 and AMRVs from North East China and Korea (H5, B78, and SC-1) were slightly lower (90.1–90.5% nucleotide identity and 98.8–99.5% amino acid identity) (Table 3). The nucleotide identities of the S segment between AMRV and other hantaviruses such as HTNV, SEOV, and PUUV were much lower at 82.6–85.8%, 73.8–74.8%, and 63.9–64.3%, respectively. The M and L segments of AP209 were closest to the identities of AMRVs (M segment, 87.5–93.6% and 96.7–98.9%; L segment, 88.9–89.4% and 98.7–99.0% for nucleotide and amino acid identities, respectively) (Table 3) among hantaviruses. Thus, isolate AP209 was identified as AMRV.

The nucleotide and amino acid sequences of all genome segments of AA57 were quite similar to the sequences of HTNV strain Bao14, which was isolated from an HFRS patient in Heilongjiang, China (S segment, 98.4% and 99.8%; M segment, 97.0% and 99.0%; L segment, 97.3% and 99.7% nucleotide and amino acid identities, respectively) (Table 3). Thus, isolate AA57 was identified as HTNV. Additionally, a comparison of the M segments between AA57 and hantavirus sequences from HFRS patients in the Khabarovsk region (KHB/HFRS/PAT#A2, KHB/HFRS/PAT#A3, and KHB/HFRS/PAT#A12)<sup>12</sup> showed 96–99% identity at the nucleotide level. The nucleotide identities of the S, M, and L segments between AA57 and other HTNVs (76-118, A9, and Z10) ranged from 85.5% to 88.8%, from 84.0% to 87.6%, and from 83.3% to 88.0%, respectively (Table 3).

Phylogenetic analyses of hantavirus S, M, and L segments were performed (Figure 2). Previously, the work by Zou and others<sup>30</sup> described that the Hantaan superclade, including HTNV and AMRV, was divided into several lineages, and the S, M, and L segments of AMRV occupied lineages S6, M6, and L5, respectively. In the present study, the S, M, and



TABLE 3

Nucleotide (ORF region) and amino acid identities\* among the two hantavirus isolates (Khekhtsir AP209 and Galkino AA57) and other hantaviruses

Strain	AMRV						HTNV					SEOV		PUUV
	Khekhtsir AP209	Solovey AP61	Solovey AP63	H5	B78	SC-1	Galkino AA57	Bao14	76-118	A9	Z10	SR-11	80-39	Sotkamo
<b>S segment</b>														
Khekhtsir AP209	–	96.3	96.0	90.1	90.1	90.5	85.1	85.8	83.0	83.0	83.6	74.8	74.1	63.9
Solovey AP61	99.8	–	99.1	90.2	90.2	90.4	84.5	85.2	82.7	82.8	83.5	73.9	74.1	64.1
Solovey AP63	99.3	99.5	–	90.1	90.1	90.4	84.2	84.9	82.8	82.6	83.4	73.8	74.0	64.3
H5	98.8	99.1	98.6	–	100.0	91.5	83.8	83.9	83.2	82.9	83.4	74.6	73.9	64.2
B78	98.8	99.1	98.6	100.0	–	91.5	83.8	83.9	83.2	82.9	83.4	74.6	73.9	64.2
SC-1	99.5	99.8	99.3	98.8	98.8	–	83.7	84.1	83.0	84.1	84.1	74.1	73.8	64.0
Galkino AA57	97.7	97.9	97.4	97.4	97.4	97.7	–	98.4	88.8	85.5	86.4	74.3	74.7	63.5
Bao14	97.4	97.7	97.2	97.7	97.7	97.4	99.8	–	89.0	86.2	86.7	74.5	74.7	63.2
76-118	96.5	96.7	96.3	96.7	96.7	96.5	98.8	99.1	–	84.8	85.8	74.3	74.6	63.0
A9	96.0	96.3	95.8	96.3	96.3	96.0	97.4	97.7	96.7	–	89.5	75.0	74.5	64.2
Z10	97.0	97.2	96.7	97.2	97.2	97.0	97.9	98.1	97.2	97.2	–	75.0	75.1	62.5
SR-11	82.1	82.1	81.6	81.6	81.6	81.8	82.3	82.1	82.3	80.9	81.1	–	97.6	62.0
80-39	83.0	83.0	82.5	82.5	82.5	82.8	83.2	83.0	83.2	81.8	82.1	98.4	–	62.0
Sotkamo	60.5	60.5	60.3	60.7	60.7	60.3	60.5	60.7	60.7	59.8	60.0	61.7	61.9	–
<b>M segment</b>														
Khekhtsir AP209	–	–	–	93.6	93.1	87.5	81.2	81.2	81.1	80.3	80.1	72.3	71.8	59.3
H5	98.9	–	–	–	96.5	87.0	79.9	79.9	79.8	79.4	79.8	71.7	71.2	60.1
B78	98.7	–	–	99.1	–	87.0	80.4	80.4	79.6	79.7	79.5	71.6	71.3	59.9
SC-1	96.7	–	–	96.9	97.1	–	80.6	80.3	80.5	79.4	80.0	72.2	71.7	59.4
Galkino AA57	92.4	–	–	92.7	92.3	91.7	–	97.0	87.6	84.0	85.0	72.0	71.8	60.1
Bao14	92.3	–	–	92.5	92.2	91.5	99.0	–	87.7	84.4	85.1	72.3	71.9	59.8
76-118	92.3	–	–	92.2	92.0	91.1	97.5	97.9	–	84.6	84.3	72.5	72.0	60.1
A9	91.6	–	–	91.5	91.2	90.6	94.7	95.0	95.4	–	87.3	71.8	71.6	60.4
Z10	92.0	–	–	91.9	91.5	90.7	94.9	95.0	95.3	96.2	–	71.6	71.6	60.0
SR-11	76.5	–	–	76.8	76.3	76.4	76.6	76.8	77.0	76.3	76.4	–	96.5	60.8
80-39	76.8	–	–	77.0	76.4	76.6	76.8	77.0	77.1	76.3	76.4	98.9	–	60.7
Sotkamo	54.3	–	–	54.5	54.4	53.9	53.8	53.6	53.8	53.6	53.7	53.8	53.8	–
<b>L segment</b>														
Khekhtsir AP209	–	–	–	89.4	–	88.9	81.9	81.9	81.5	81.6	82.2	–	75.6	67.5
H5	99.0	–	–	–	–	88.6	81.6	81.2	81.7	81.0	81.8	–	75.4	67.7
SC-1	98.7	–	–	98.7	–	–	95.9	81.1	81.3	81.1	81.4	–	75.3	67.4
Galkino AA57	96.1	–	–	96.0	–	81.2	–	97.3	88.0	83.4	84.0	–	74.9	67.3
Bao14	96.1	–	–	95.9	–	95.8	99.7	–	88.1	83.2	84.0	–	74.8	67.2
76-118	95.7	–	–	95.8	–	95.6	98.7	98.6	–	83.6	83.5	–	74.3	66.8
A9	94.2	–	–	94.3	–	93.9	95.8	95.6	95.6	–	85.4	–	74.7	67.1
Z10	95.5	–	–	95.5	–	95.3	97.6	97.5	97.3	96.1	–	–	74.9	67.3
80-39	85.5	–	–	85.3	–	85.3	85.4	85.4	85.0	83.7	85.4	–	–	67.7
Sotkamo	69.1	–	–	68.7	–	68.7	69.1	69.0	68.9	67.9	69.3	–	68.5	–

\* Values to the right above the diagonal show nucleotide identities; values to the left below the diagonal show amino acid identities.

L segments of AP209 clustered with AMRVs in lineages S6, M6, and L5, respectively, consistent with the results in the work by Zou and others.<sup>30</sup> AP209 was more related to AMRVs detected in the Primorsky region of Far East Russia, such as AP708, AP1371, AP1168, Solovey/AP61/1999, and Solovey/AP63/1999, than AMRVs from China and Korea. Although the M and L segments of AMRV clustered in distinct lineages from other HTNVs, the S segment of AMRV clustered within HTNV. The S, M, and L segments of strain AA57 clustered with HTNVs from the Primorsky region (AA1028, AA1719, and AA2499) and HTNVs from China (Bao14 and CGHu1), which consist of the Far East (FE) genotype within HTNVs.<sup>13,28</sup> Interestingly, strain CGHu1 isolated from Guizhou, a southern province of China, also clustered with the FE genotype. HTNV lineages in S1 were divided into S1-a and S1-b sublineages according to their geographical origins: S1-a from Korea (76-118, CUMC-B11, and Maaji-2) and S1-b from Far East Russia (AA57, AA1028, AA1719, and AA2499) and China (Bao14 and CGHu1). The same clustering patterns were also observed with the M and L segments.

**Antigenic characterization.** The antigenic characterization of AP209, AA57, and other hantaviruses was performed using

a neutralization test (Table 4). Anti-AMRV mouse sera had more than fourfold higher titers against AMRV strains H5 and AP209 than those titers against HTNV and SEOV. Anti-AA57 mouse serum had a more than fourfold higher neutralization titer against AA57 compared with those titers against AMRVs and SEOV. These results indicate that the antigenicities of AMRV, HTNV, and SEOV are different and that the neutralization test may be useful in the differential diagnosis of HFRS caused by AMRV, HTNV, or SEOV in Far East Russia.

**Serological analysis of patient sera.** To examine the causative agents of HFRS in the Khabarovsk and Primorsky regions, the neutralization test was performed on a total of 67 HFRS patients who had IFA antibodies to hantaviruses (Tables 5 and 6). The hantavirus in each HFRS patient was determined as a certain virus giving a neutralization titer more than or equal to fourfold higher than the titer of other viruses (Tables 5 and 6). If the neutralization titer difference was less than fourfold, we considered the infected hantavirus of the sample to be untyped. If the neutralization titer was less than 1:20 for any hantaviruses, we regarded the sample as negative for hantavirus infection. Of 67 HFRS patients, 14, 13, and 15 were



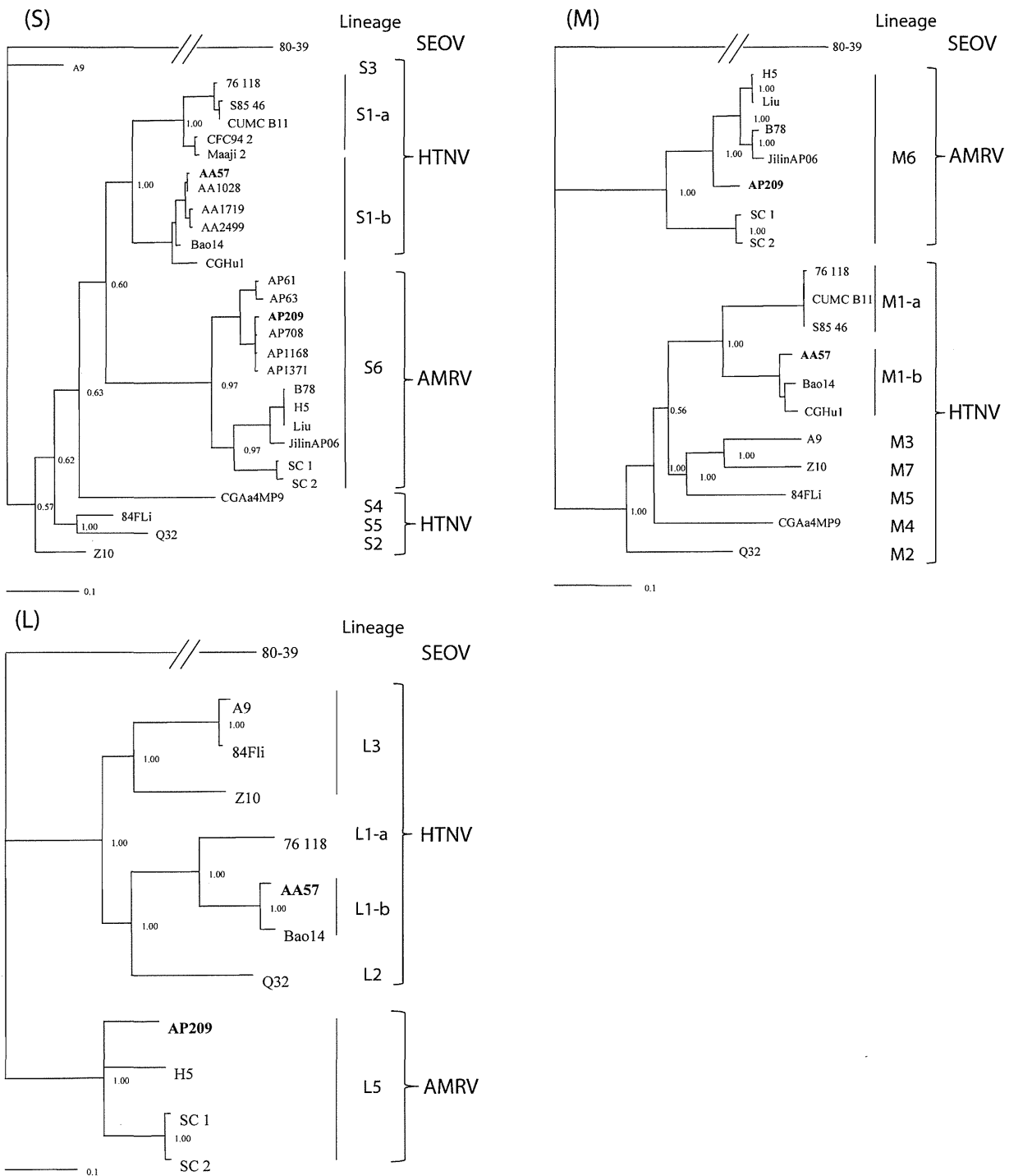


FIGURE 2. Phylogenetic consensus trees of hantaviruses based on nucleotide sequences covering the entire ORF of the S, M, and L segments. The trees were generated using MCMCMC analyses. The reliability of the tree was evaluated by the posterior probability value derived from MrBayes. The scale bar indicates 0.1 nucleotide substitutions per site.

diagnosed as AMRV (20.9%), HTNV (19.4%), and SEOV (22.4%) infections, respectively, and 25 patients (37.3%) were untyped (Tables 5 and 6). All of 20 untyped sera in Khabarovsk region showed strong cross-reaction to AMRV and HTNV (Table 6). The seroprevalence of AMRV, HTNV, and SEOV in HFRS patients differed with each sampling site ( $\chi^2$  value = 70.69, degrees of freedom [df] = 6,  $P < 0.001$ ). SEOV (60.0%;  $P < 0.001$ ) and AMRV (81.8%;  $P < 0.001$ ) were the major causative agents of HFRS in Vladivostok city and the rural

areas of Primorsky region except Vladivostok, respectively (Table 5). HTNV (35.5%;  $P < 0.002$ ) may be one of the major causes in the Khabarovsk region (Table 6).

DISCUSSION

Previous investigations of HFRS patients in Far East Russia have revealed that AMRV, HTNV, and SEOV are causative agents of HFRS in this region.<sup>12,13</sup> However, no complete

TABLE 4

Antigenic characterization of Khekhstir AP209 and Galkino AA57 by cross-neutralization test using immune mouse sera

Virus	Antisera Strain	Neutralization titer*			
		AMRV H5	HTNV Khekhstir AP209	HTNV Galkino AA57	SEOV SR-11
AMRV	H5	80	80	20	< 20
AMRV	Khekhstir AP209	320	640	40	20
HTNV	Galkino AA57	640	640	2,560	40
SEOV	SR-11	< 20	< 20	< 20	40

\* Neutralization titer was expressed as a reciprocal of the highest dilution that showed 80% or more inhibition of virus focus formation.

etiological analysis of each HFRS patient to identify the infected hantavirus has been conducted. Because the biological properties of hantaviruses in this region have not been well-characterized, a differential diagnosis of infected viruses in HFRS is quite difficult. Thus, we isolated hantaviruses in Far East Russia and characterized the new isolates. Additionally, serological differential diagnoses were carried out on HFRS patients in this region to identify the virus in each infected patient.

TABLE 5

Differential diagnosis of HFRS patients in the Primorsky region by neutralization test

Patient number	Days after onset	Neutralization titer			Infected virus
		AMRV Khekhstir AP209	HTN Galkino AA57	SEOV SR-11	
Vladivostok city					
1	14	< 20*	< 20	40	SEOV
2	11	< 20	< 20	40	SEOV
3	11	20	20	80	SEOV
4	17	20	< 20	160	SEOV
5	16	40	< 20	40	UT†
6	8	< 20	< 20	40	SEOV
7	9	40	20	160	SEOV
8	12	40	40	320	SEOV
9	25	640	80	20	AMRV
10	26	20	20	160	SEOV
11	14	< 20	40	< 20	HTNV
12	20	20	20	80	SEOV
13	11	< 20	40	< 20	HTNV
14	28	< 20	80	80	UT
15	17	20	20	80	SEOV
16	17	1,280	80	< 20	AMRV
17	730	40	20	160	SEOV
18	60	80	20	< 20	AMRV
19	17	< 20	< 20	80	SEOV
20	8	< 20	< 20	40	SEOV
21	10	20	< 20	80	SEOV
22	28	< 20	< 20	40	SEOV
23	8	20	< 20	20	UT
24	13	320	< 20	< 20	AMRV
25	26	1,280	80	20	AMRV
Outside Vladivostok					
26	20	1,280	160	20	AMRV
28	6	320	40	20	AMRV
29	5	1,280	320	40	AMRV
30	13	160	40	< 20	AMRV
32	9	1,280	160	20	AMRV
33	32	80	160	< 40	UT
35	17	1,280	160	40	AMRV
36	15	640	80	< 20	AMRV
37	20	640	160	40	AMRV
38	16	< 40	< 40	40	UT
39	22	1,280	320	20	AMRV

\* 80% focus reduction method.

† Untyped.

TABLE 6

Differential diagnosis of HFRS patients in the Khabarovsk region by neutralization test

Patient no.	Months after onset	Neutralization titer			Infected virus
		AMRV Khekhstir AP209	HTNV Galkino AA57	SEOV SR-11	
1	142	40	160	20	HTNV
2	71	40	80	< 20	UT
3	48	160	160	20	UT
5	13	320	1,280	< 20	HTNV
6	5	160	320	40	UT
7	18	160	160	20	UT
8	17	160	640	40	HTNV
9	3	80	320	20	HTNV
10	4	80	160	20	UT
11	144	320	320	40	UT
12	6	160	160	< 20	UT
13	3	40	160	< 20	HTNV
14	5	80	320	20	HTNV
15	5	160	320	20	UT
16	4	40	80	< 20	UT
17	13	80	160	< 20	UT
18	3	80	160	< 20	UT
19	3	40	160	< 20	HTNV
20	4	1,280	5,120	40	HTNV
21	4	80	160	20	UT
22	4	160	160	20	UT
23	4	320	1,280	20	HTNV
24	2	320	320	< 20	UT
25	1	80	320	< 20	HTNV
26	2	80	80	< 20	UT
27	396	80	320	20	HTNV
28	2	160	160	< 20	UT
29	1	80	160	< 20	UT
30	2	160	320	< 20	UT
31	168	160	160	< 20	UT
32	288	160	320	20	UT

Rodent surveys were carried out in rural areas of the Khabarovsk region, and we confirmed that HTNV and AMRV were maintained in *A. agrarius* and *A. peninsulae*, respectively, which we have described previously for hantavirus infection in rodents of the Primorsky region, Russia.<sup>8,11</sup> These results indicated that HTNV and AMRV are distributed throughout Far East Russia.

We successfully isolated four strains of HTNV and one strain of AMRV. Among the isolates, HTNV strain AA57 from *A. agrarius* and AMRV strain AP209 were subjected to genetic and antigenic characterization. AA57 was genetically closely related to the sequences from HFRS patients in the Khabarovsk region<sup>12</sup> and Bao14, which was isolated from HFRS patients in China. Newly isolated AMRV AP209 from *A. peninsulae* was genetically closely related to the sequences from rodents and HFRS patients in the Primorsky region. These results indicate that HTNV and AMRV are the causative agents of HFRS in the Khabarovsk region. Although the M and L segments of AMRV and HTNV clustered in distinct lineages, the S segment of AMRV was included in HTNV. This observation was also reported in the work by Zou and others.<sup>30</sup> Previous reports of phylogenetic analysis have indicated that a natural reassortment occurred between HTNV and SEOV in China and among different lineages of PUUVs in nature.<sup>31,32</sup> Additionally, dual infection with Sin Nombre virus and Black Creek Canal virus could produce reassortant viruses under experimental conditions.<sup>33</sup> Other reports have described that a recombination of the virus genome occurred in Dobrava virus and Saaremaa virus and in different lineages of Tula

viruses in nature.<sup>34,35</sup> Thus, it is feasible that some discontinuous mutation, such as reassortment, may have occurred between AMRV and HTNV as indicated between HTNV and SEOV in China.<sup>32</sup>

In the cross-neutralization test using immune sera against HTNV, AMRV, and SEOV, the immune sera had fourfold higher titers against the homologous virus than the heterologous viruses. This result indicates that the cross-neutralization test is useful for the differential diagnosis of AMRV, HTNV, and SEOV infection in Far East Russia.

To obtain more information about HFRS etiology and epidemiology in Far East Russia, the neutralization tests were carried out using HFRS patient sera from the Primorsky and Khabarovsk regions. The results showed that AMRV (20.9%), HTNV (19.4%), and SEOV (22.4%) were the causative agents of HFRS in Far East Russia, and these findings are consistent with the results of previous reports.<sup>13</sup> However, the major cause of HFRS varied according to location. SEOV was the most common cause of HFRS in Vladivostok city in the Primorsky region (15/25). In Vladivostok city, *R. norvegicus* and *R. rattus* may transmit SEOV to city residents (Table 5). Some patients in Vladivostok were infected with AMRV and HTNV (5/25 and 2/25, respectively). Russian people frequently visit vegetable gardens in rural areas called dachas to cultivate plants. Because *A. peninsulæ* and *A. agrarius* may live in forests and fields near dachas, it is highly possible that people, including city residents, may acquire AMRV or HTNV infection close to dachas. Interestingly, in the rural area of the Primorsky region, most patients were infected with AMRV (9/11) (Table 5). It is possible that people in this area may become infected more frequently with AMRV in forests than *A. peninsulæ* inhabit. However, in the Khabarovsk region, HTNV may be the major cause of HFRS (Table 6). AMRV infection may occur in the Khabarovsk region, although no AMRV-specific antibodies were detected in this study. There are three possible explanations for negative results of AMRV-specific antibodies. (1) Residents in Khabarovsk region may contact more frequently with *A. agrarius* than *A. peninsulæ*. (2) Because of the similar antigenicity between AMRV and HTNV, AMRV-infected patients may produce antibodies cross-reacted to HTNV. Most patient sera from the Khabarovsk region were collected in late convalescent phase (Table 6), which may have influenced the results of neutralization test. (3) The possibility of dual infections with AMRV and HTNV cannot be ruled out. However, because *A. agrarius* and *A. peninsulæ* preferentially inhabit grass field and forest, respectively, the possibility of the dual infection at the same time may be extremely low. In addition, consecutive infections of the two viruses may not occur, because patients infected with AMRV or HTNV can produce cross-neutralizing antibodies to the homologous and heterologous viruses. Additional studies should be conducted to develop a clear picture of HFRS epidemiology on a larger scale. Unfortunately, in some patients (25/71), we could not differentiate the infecting hantavirus. Serotype-specific conformational epitopes have been detected in the center and C-terminal regions of the N.<sup>24,36</sup> Thus, a serotyping system using N-terminal truncated N could be a solution for the differential diagnosis of hantavirus infections.<sup>37,38</sup>

In conclusion, new isolates of AMRV and HTNV were characterized, and AMRV, HTNV, and SEOV infections were differentiated in HFRS patients of Far East Russia. The major cause of HFRS varied by location in the region: SEOV for

Vladivostok city in the Primorsky region, AMRV in the rural areas of the Primorsky region, and probably HTNV for the Khabarovsk region. Additional study is necessary to determine the risk factors of HFRS for each pathogenic virus in Far East Russia.

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## Genetic diversity of hantaviruses in Mexico: Identification of three novel hantaviruses from Neotominae rodents

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### ABSTRACT

A variety of hantaviruses are harbored by rodents in North and South America, some of which can cause hantavirus pulmonary syndrome. To obtain greater evolutionary insight into hantaviruses in the Americas, a total of 211 rodents were captured in the Mexican states of Guerrero and Morelos in 2006. Anti-hantavirus antibodies were detected in 27 of 211 serum samples (12.8%) by ELISA. The distribution of seropositive rodents was: 17 *Peromyscus beatae*, 1 *Megadontomys thomasi*, 1 *Neotoma picta*, 6 *Reithrodontomys sumichrasti*, and 2 *Reithrodontomys megalotis*. The hantavirus small (S), medium (M), and large (L) genome segments from *P. beatae*, *R. sumichrasti*, and *R. megalotis* were amplified and the sequences covering the open reading frames were determined. The hantaviruses from *P. beatae*, *R. sumichrasti*, and *R. megalotis* were provisionally designated Montano (MTN), Carrizal (CAR), and Huitzilac (HUI), respectively. The M segment amino acid identities among the Mexican hantaviruses were 80.8–93.0%. When these M segments were compared to those of known hantaviruses, MTN virus was most closely related to Limestone Canyon (LSC) virus (88.9% amino acid identity), while the CAR and HUI viruses were most closely related to El Moro Canyon (ELMC) virus (90–91% identity). Phylogenetic analysis revealed that the MTN, CAR, and HUI viruses occupy a monophyletic clade with the LSC, ELMC, and Rio Segundo viruses, which are harbored by *Peromyscus boylii*, *R. megalotis*, and *Reithrodontomys mexicanus*, respectively. The data obtained in this study provide important information for understanding the evolution of hantaviruses in the Americas.

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### 1. Introduction

Hantaviruses, which comprise the genus *Hantavirus* in the family *Bunyaviridae*, contain a three-segment negative-sense RNA genome. Small (S), medium (M), and large (L) RNA segments encode the nucleocapsid protein (N), glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase, respectively (Schmaljohn et al., 1986). Hantaviruses are carried by a variety of rodents and soricomorph species in nature and persistently infect their natural hosts (Jonsson et al., 2010; Song et al., 2007). Some rodent-borne hantaviruses cause hantavirus infections in humans, including hemorrhagic fever with renal syndrome (HFRS) and hantavirus

pulmonary syndrome (HPS) (Peters et al., 1999; Schmaljohn and Hjelle, 1997). HFRS, which is characterized by a high fever, hemorrhage, and renal dysfunction with a fatality rate <10%, is distributed throughout Europe, Russia, China, and Korea. HPS, which is characterized by a high fever, pulmonary dysfunction, and cardiac shock and has a ~40% fatality rate (Peters and Khan, 2002), is distributed in North and South Americas. Humans acquire the infection primarily by the inhalation of virus-containing rodent excreta or by the bite of infected animals (Lee and Johnson, 1982). An important characteristic of hantavirus is the close relationship between the virus and its rodent host species, and co-evolution between them may have occurred on a geological timescale (Plyusnin and Morzunov, 2001).

Since the identification of HPS in the United States of America, patients have been reported from various countries in both North and South America; more than 30 hantaviruses have been

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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 palustris*, 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 Marias, 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  $-80^{\circ}\text{C}$  until use. Filter paper was used to collect blood samples from dead animals. The serum and clots were separated by centrifugation at  $12,000 \times g$  for 5 min. The sera were heat-inactivated at  $56^{\circ}\text{C}$  for 30 min and stored at  $-40^{\circ}\text{C}$  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  $-80^{\circ}\text{C}$  until required. SNV-rN was added to 96-well plates (50  $\mu\text{l}$ /well) and kept at  $4^{\circ}\text{C}$  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  $37^{\circ}\text{C}$  for 1 h. After washing, 1:100 dilutions of wild rodent sera were applied to the plates (50  $\mu\text{l}$ /well) and incubated at  $37^{\circ}\text{C}$  for 1 h. The plates were washed and incubated with 50  $\mu\text{l}$ /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  $\mu\text{l}$ /well) at  $37^{\circ}\text{C}$  for 1 h. After washing, 200  $\mu\text{l}$  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  $\mu\text{g}/\text{ml}$  in sample buffer [1% sodium dodecyl sulfate (SDS), 1%  $\beta$ -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<sup>2</sup> 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  $4^{\circ}\text{C}$  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  $-80^{\circ}\text{C}$  until they were used for RNA extraction. Total RNA was extracted according to the manufacturer's instructions. RNA (2  $\mu\text{g}$ ) 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

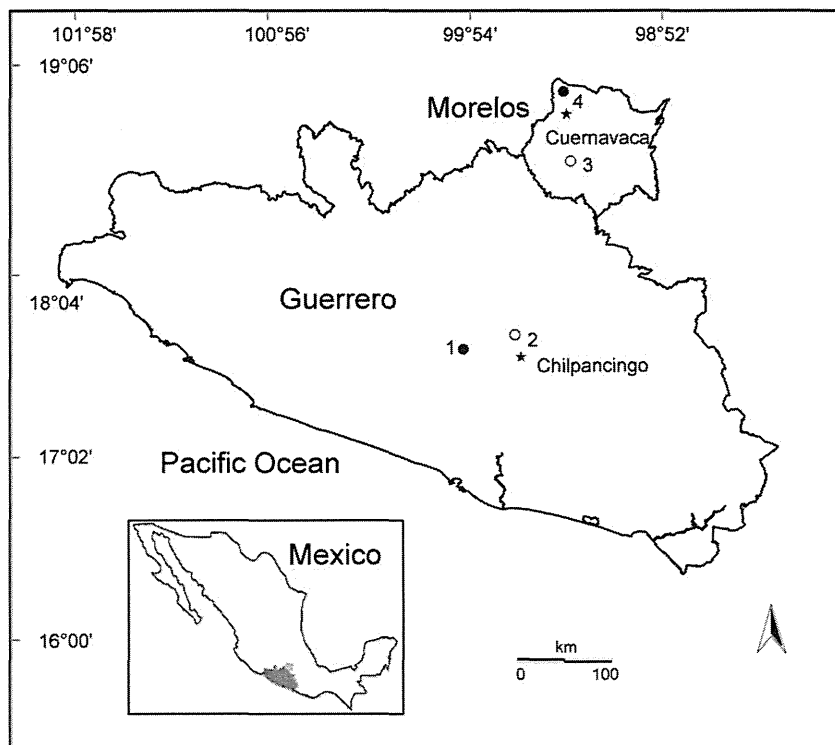
### 3.1. Investigation of hantavirus infection in Mexican rodents

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 Huitzilac, 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 Marias (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	
<b>Leonardo Bravo, Guerrero</b>				
<i>Baiomys musculus</i>	1	0		0
<i>Peromyscus beatae</i>	50	17 (34.0)	16	16 (32.0)
<i>Peromyscus lepturus</i>	5	0		0
<i>Peromyscus megalops</i>	24	0		1 (4.2)
<i>Peromyscus mexicanus</i>	10	0		0
<i>Peromyscus</i> spp.	14	0		0
<i>Megadontomys thomasi</i>	6	1 (16.7)	1	0
<i>Neotoma picta</i>	6	1 (16.7)	1	0
<i>Reithrodontomys sumichrasti</i>	14	6 (42.9)	6	3 (21.4)
Subtotal	130	25 (19.2)	24	20 (15.4)
<b>Eduardo Neri, Guerrero</b>				
<i>Hodomys alleni</i>	4	0		0
<i>Osgoodomys banderanus</i>	1	0		0
<i>Peromyscus melanophrys</i>	3	0		0
<i>Reithrodontomys sumichrasti</i>	1	0		0
Subtotal	9	0		0
<b>Zacatepec, Morelos</b>				
<i>Mus musculus</i>	10	0		0
<i>Liomys irroratus</i>	2	0		0
Subtotal	12	0		0
<b>Huitzilac and Tres Mariás, Morelos</b>				
<i>Microtus mexicanus</i>	15	0		0
<i>Mus musculus</i>	2	0		0
<i>Neotoma mexicana</i>	1	0		0
<i>Neotomodon alstoni</i>	2	0		0
<i>Peromyscus hylocetes</i>	13	0		0
<i>Peromyscus maniculatus</i>	2	0		0
<i>Reithrodontomys megalotis</i>	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*

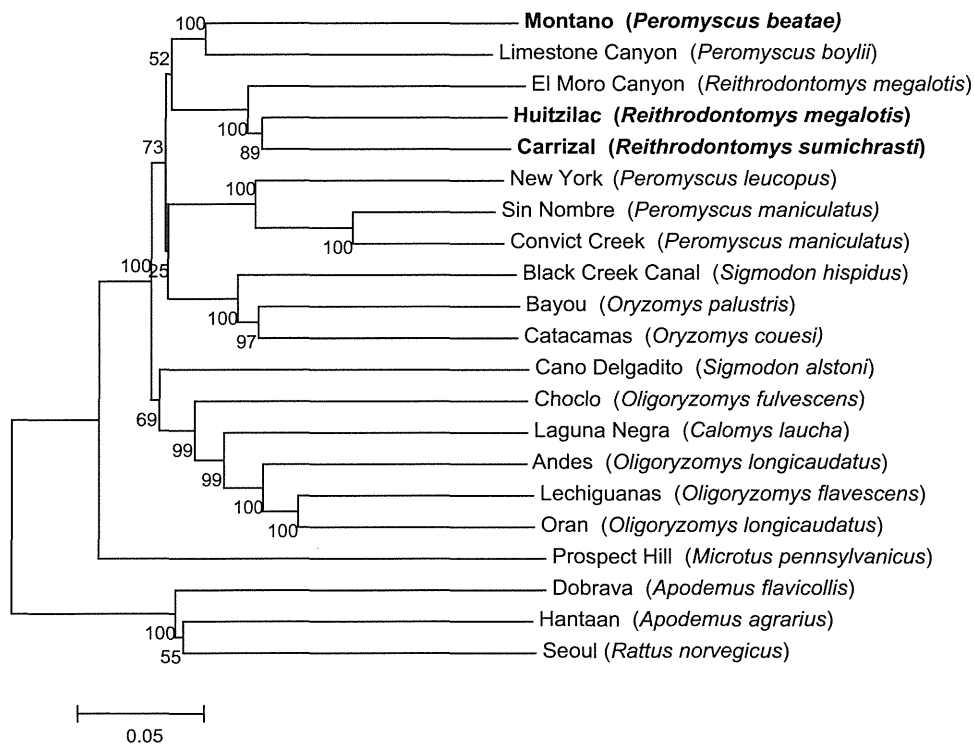
**Table 2**  
Nucleotide and amino acid identities of S segment entire coding region among newly recognized Mexican and other hantaviruses.

	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 (%)															



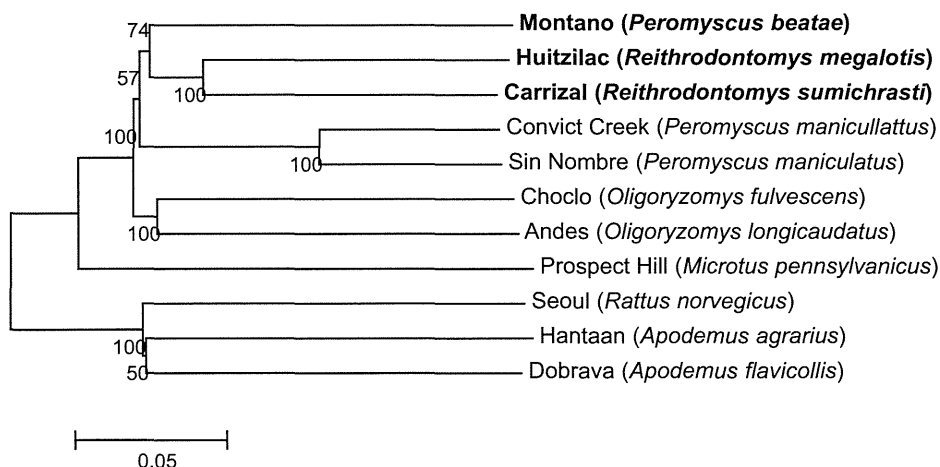


**Fig. 3.** Phylogenetic tree of the hantavirus M gene. Hantavirus M genes (from 52 to 3474 nucleotides corresponding to Sin Nombre virus) were used to construct the phylogenetic tree. A software package for genetic analysis, MEGA 4 (<http://www.megasoftware.net/mega.html>), was used to generate multiple 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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