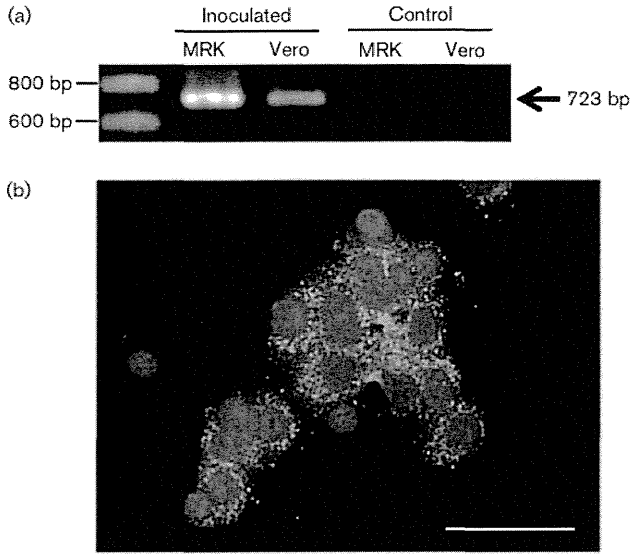

Fig. 4. Confirmation of the isolation of HOKV. (a) Detection of the HOKV genome in tissue-inoculated MRK101 and Vero E6 cells at 14 days p.i. by RT-PCR. HOKV-specific products (723 bp) were amplified from RNA extracted from inoculated MRK101 and Vero E6 cells. (b) Immunofluorescence of tissue-inoculated MRK101 cells at 14 days p.i. Inoculated MRK101 cells were stained for hantavirus N protein (green) and nuclei (blue). Bar, 50 μm.

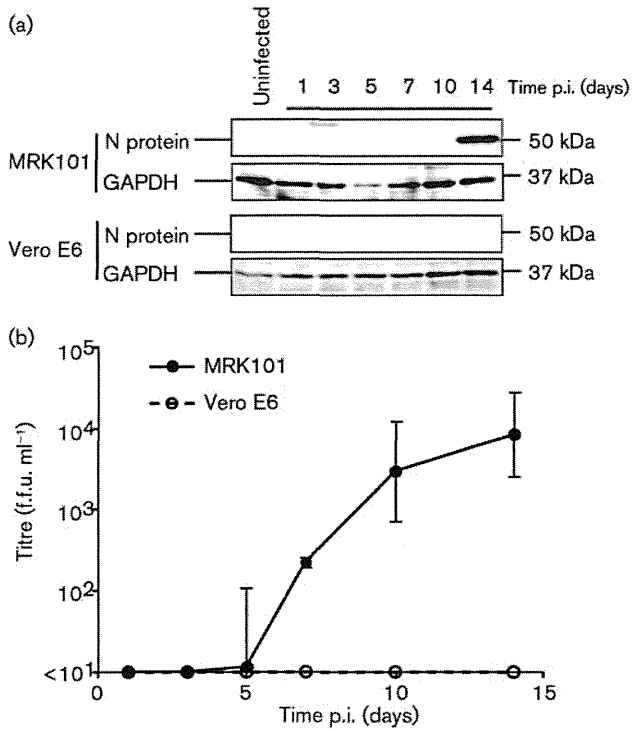

Fig. 5. MRK101 cells and Vero E6 cells infected with HOKV strain Kitahiyama128/2008. (a) Expression of viral N protein in HOKV-infected MRK101 and Vero E6 cells. (b) Virus titres in culture fluid of HOKV-infected MRK101 and Vero E6 cells. Error bars represent SD.

Phylogenetic analysis based on the nucleotide sequence of the coding region of the S (Fig. 6a), M (Fig. 6b) and L (Fig. 6c) segments supported the close relationship between HOKV and PUUV. However, it also indicated that HOKV is located on a separate branch from PUUV. The topologies of the phylogenetic trees for the S, M and L segment sequences of HOKV were near-identical, suggesting that all three HOKV segments have a similar evolutionary history during which no heterologous reassortment occurred.

DISCUSSION

Hantaviruses are emerging pathogens, some of which cause life-threatening infections. Vero E6 cells have been used for hantavirus research for many years because of their susceptibility to these viruses, but propagation of some hantaviruses in this cell line is problematic. Therefore, several hantaviruses have not yet been isolated and only a limited number have been characterized. The purpose of this study was to establish a cell line for the *in vitro* study of hantaviruses, particularly their isolation and propagation. In previous research, primary kidney cells derived from bank voles, the natural host of PUUV, showed a high susceptibility to PUUV (Temonen *et al.*, 1993), suggesting that hantaviruses propagate well in cells derived from the natural host of the virus. Herein, we established a cell line, MRK101, derived from the kidney of the grey red-backed vole (the natural host of HOKV), and demonstrated its susceptibility to hantaviruses. Using this cell line, HOKV was isolated successfully.

In the present study, MRK101 cells derived from the *Myodes* rodent showed a high susceptibility to PUUV, a *Myodes*-borne hantavirus, moderate susceptibility to SEOV, a *Rattus*-borne hantavirus, and low or no susceptibility to AMRV and HTNV, *Apodemus*-borne hantaviruses. These data suggest that the susceptibility level is associated with the host species of each hantavirus. In a previous *in vivo* study, the susceptibility of animals to hantaviruses correlated well with the genetic relationships of the natural reservoirs (Klingström *et al.*, 2002), which is consistent with our findings. Other rodents, such as members of the genus *Microtus* and the subfamily Sigmodontinae, are genetically closer to *Myodes* rodents than rodents in the subfamily Murinae such as *Apodemus* and *Rattus* species (Vapalahti *et al.*, 1999). Therefore, MRK101 cells may be susceptible to *Microtus*-borne hantaviruses such as Tula virus (TULV) and Prospect Hill virus (PHV) and New World hantaviruses such as SNV and ANDV. Further study of the susceptibility of MRK101 cells is needed.

The factors that determine hantavirus propagation in cell culture remain unknown. One possibility is an interaction between host and viral proteins. To date, various host proteins have been reported to be associated with viral proteins in the hantavirus life cycle. Integrins are considered to be the receptors for the viral glycoprotein, allowing entry

Table 1. Identities of nucleotide (ORF region) and amino acid sequence between HOKV strain Kitahiyama128/2008 and other hantaviruses

Virus	Strain	Identity (%) of HOKV strain Kitahiyama128 with:					
		S segment nucleotides	S segment amino acids	M segment nucleotides	M segment amino acids	L segment nucleotides	L segment amino acids
HOKV	Kamiiso	98.7	100.0	–*	–	–	–
	Tobetsu	97.0	99.8	–	–	–	–
PUUV	CG1820	81.9	94.7	78.5	90.7	79.7	95.5
	Samara49	82.5	95.2	79.7	91.6	79.7	95.5
	Samara94	82.4	95.2	79.9	91.5	79.7	95.3
	Sotkamo	83.8	95.8	78.1	90.6	80.7	95.3
	DTK/Ufa-97	82.0	94.9	78.7	91.3	79.8	95.6
TOPV	Ls136V	76.1	88.2	74.1	84.3	–	–
KBRV	MF-43	76.0	88.0	74.2	82.6	–	–
TULV	5302v	72.7	80.1	72.9	80.5	74.9	86.1
PHV	PH-1	70.3	80.1	70.7	77.3	73.0	84.0
SNV	NMR11	70.1	71.4	66.4	67.5	70.6	78.0
HTNV	76-118	64.0	61.2	60.0	55.2	66.5	69.6
AMRV	AP209	62.6	60.7	60.1	55.8	66.6	69.6
SEOV	80-39	65.4	62.1	59.7	53.8	66.5	69.2

*Comparison not done.

into the cell (Gavrilovskaya *et al.*, 1998, 1999). The GPI (glycosylphosphatidylinositol)-anchored protein decay-accelerating factor (DAF)/CD55, the gC1qR/p32 protein, and various other cellular proteins have also been shown to mediate hantavirus infection (Choi *et al.*, 2008; Kim *et al.*, 2002; Krautkrämer & Zeier, 2008; Mou *et al.*, 2006). In addition, various cellular proteins, such as the ribosomal S19 protein and Daxx, have been reported to interact with the hantavirus N protein (Haque & Mir, 2010; Lee *et al.*, 2003; Li *et al.*, 2002; Maeda *et al.*, 2003). Variation in these host proteins among species may affect their interaction with viral proteins, resulting in the observed different propagation patterns. The Vero E6 cell line-adaptation of hantaviruses may also affect their propagation in cell culture. Hantaviruses used in this study, with the exception of HOKV, were isolated and cultured in Vero E6 cells and are considered to be adapted to this cell line. Since Vero E6 cells are IFN-α/β-deficient and are derived from the African green monkey, adaptation to Vero E6 cells has the potential to affect virus properties. Indeed, a previous study showed that full adaptation of PUUV to Vero E6 cells reduced its ability to infect its natural host compared with the parental wild-type virus (Lundkvist *et al.*, 1997). Adaptation to Vero E6 cells may affect the infectivity of hantaviruses to host rodent cells. To evaluate the effect of adaptation to Vero E6 cells, comparative analysis of the characteristics of hantaviruses isolated in Vero E6 cells and those isolated in MRK101 cells or other host cells is required. A study that aims to clarify whether adaptive mutation of hantavirus occurs in MRK101 cells is now in progress.

Since its identification in 1995, we have attempted to isolate HOKV using Vero E6 cells, but all efforts were unsuccessful (Kariwa *et al.*, 1995). In this study, we successfully isolated HOKV using MRK101 cells. Since almost all hantaviruses were isolated using Vero E6 cells, this is to our knowledge the first report of hantavirus isolation from a cell line derived from the natural host of the virus. Although numerous hantaviruses have been identified, many were detected only by the presence of the genome and have not yet been isolated (Arai *et al.*, 2008; Song *et al.*, 2007). Use of MRK101 cells or other cell lines derived from the natural host will probably facilitate hantavirus isolation. Notably, HOKV was successfully propagated in MRK101 but not Vero E6 cells. These results are consistent with the failure of HOKV isolation in Vero E6 cells. Our results indicate that some hantaviruses may intrinsically propagate in Vero E6 cells or are more likely to adapt to Vero E6 cells, e.g. HTNV, PUUV and SEOV. However, other hantaviruses are less likely to adapt to Vero E6 cells, e.g. HOKV. At present, the reason for the inability of HOKV to propagate in Vero E6 cells is not known, but clarification of this issue would represent useful information regarding cell adaptation, cell tropism and host specificity.

Of the hantaviruses associated with the subfamily Arvicolinae (voles and lemmings), including HOKV, PUUV, TULV and PHV, only PUUV has been reported to be associated with human disease (Kariwa *et al.*, 2007). The factors that

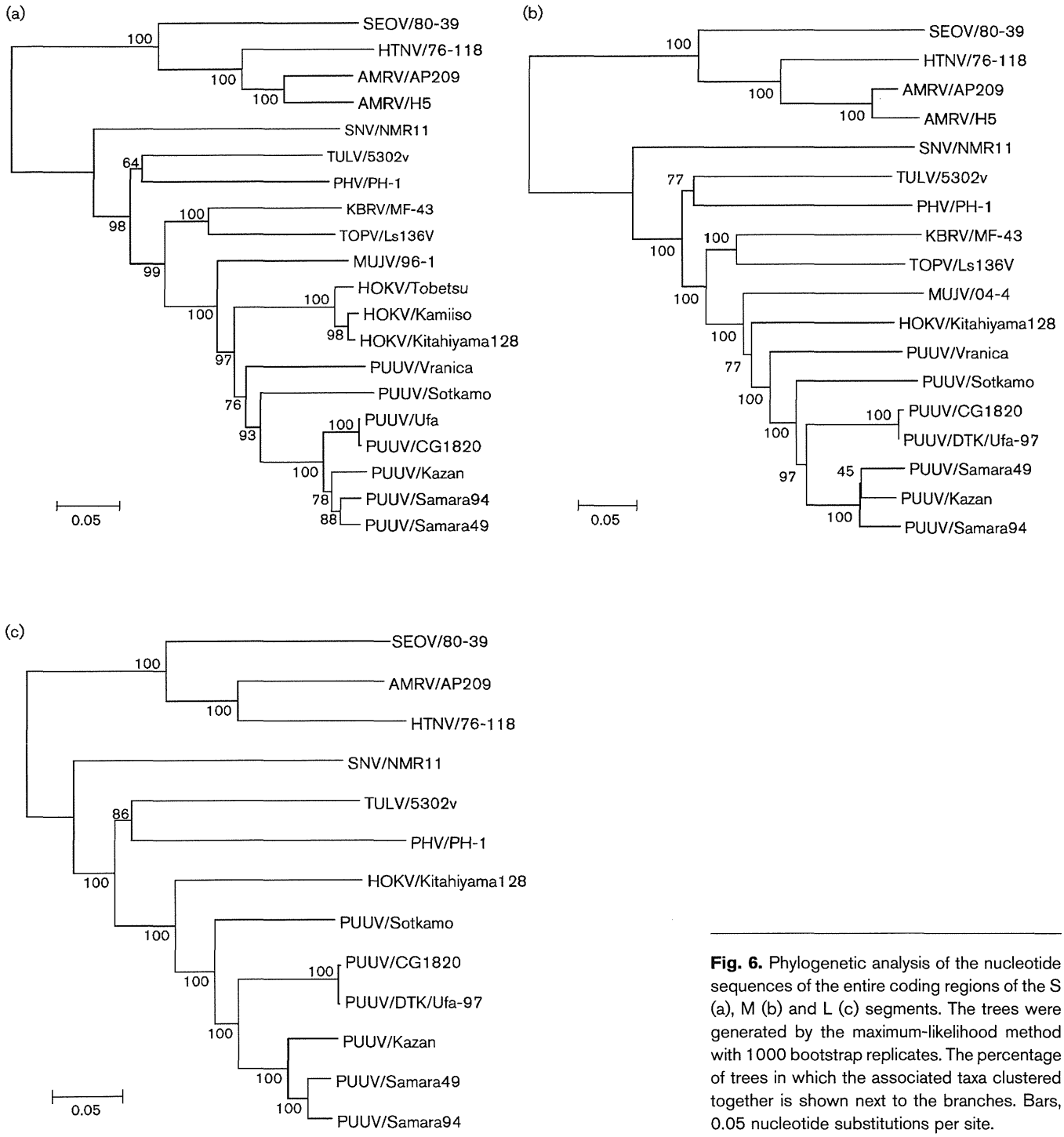

Fig. 6. Phylogenetic analysis of the nucleotide sequences of the entire coding regions of the S (a), M (b) and L (c) segments. The trees were generated by the maximum-likelihood method with 1000 bootstrap replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bars, 0.05 nucleotide substitutions per site.

determine the pathogenesis of PUUV to humans are of great interest. Phylogenetic analyses of the S, M and L segment sequences indicate that HOKV is most closely related to PUUV, in conjunction with which it has diverged from a common ancestral node. A 5–10 % amino acid difference and a ~20 % nucleotide difference exist between HOKV and PUUV. Although further studies are needed to ascertain whether HOKV causes human disease, some changes that determine pathogenicity to humans may be contained in the amino acid sequence differences between PUUV and HOKV.

Grey red-backed voles are known to be the natural hosts of several viruses, including HOKV, tick-borne encephalitis virus (TBEV) and Ljungan virus (LV) (Niklasson *et al.*, 2006; Yoshii *et al.*, 2011). We confirmed that MRK101 cells were susceptible to TBEV infection (data not shown).

Other researchers have demonstrated that cell lines derived from the bank vole are susceptible to various viruses, such as TBEV, LV and cowpox virus (Essbauer *et al.*, 2011; Stoltz *et al.*, 2011). Therefore, this cell line may facilitate the isolation and propagation of other *Myodes*-borne viruses.

In conclusion, MRK101 cells may be a useful tool for studies of hantavirus infection *in vitro*. This is to our knowledge the first report of hantavirus isolation using a cell line that originated from the natural host. MRK101 cells and HOKV will probably contribute to elucidating the mechanisms of hantavirus replication, host specificity, pathogenesis and persistent infection in wild rodents.

METHODS

Rodent capture. Grey red-backed voles were captured in the towns of Kitahiyama in November 2008, and Tobetsu in September 2010, in Hokkaido, Japan. Lungs, kidneys and serum were collected. Tissues collected in Kitahiyama were used for isolating HOKV and those collected in Tobetsu were used for preparing the cell line.

Cell cultures and viruses. Kidney tissue was collected from an adult female grey red-backed vole captured in 2010. The sample was minced and digested with 0.167 % trypsin (Becton Dickinson) at 37 °C for 1 h. Collected cells were centrifuged at 200 ***g*** for 4 min and resuspended in Dulbecco's modified Eagle's medium (DMEM, with 4.5 g l^{-1} glucose; Gibco), supplemented with 10 % FBS (MP Biomedicals), 100 IU penicillin ml^{-1} and 100 μg streptomycin ml^{-1} (both from Wako). The cell suspension was seeded in a 25 cm^2 flask and incubated at 37 °C in a 5 % CO_2 atmosphere. The cells were subcultured immediately prior to confluence. Cloning of cells was performed by the limiting dilution method. Briefly, the cells were trypsinized, dispersed well, diluted to the concentration of 5 cells ml^{-1}, and seeded into 96-well plates (0.5 cell per well). After 24 h incubation, the wells containing one cell were selected. The cells from the selected wells were cloned again and subcultured. One of the established clones was successfully cultured over 100 serial passages. The newly established cell line was designated 'MRK101' (*Myodes rufocanus* kidney).

Contamination with mycoplasma was tested by PCR using universal primers (TaKaRa PCR Mycoplasma Detection Set; Takara Bio) and by DAPI staining. Contamination with hantavirus and lymphocytic choriomeningitis virus (LCMV) were investigated by PCR and an indirect immunofluorescence antibody test (IFA), respectively, as described previously (Ike *et al.*, 2007).

Vero E6 (African green monkey kidney) cells were cultivated in Eagle's minimum essential medium (EMEM; Gibco) supplemented with 10 % FBS, 100 IU penicillin ml^{-1} and 100 μg streptomycin ml^{-1} at 37 °C in a 5 % CO_2 incubator.

Virus stocks of HTNV 76-118 (Lee *et al.*, 1978), SEOV SR-11 (Kitamura *et al.*, 1983), AMRV H5 (Lokugamage *et al.*, 2004), PUUV Sotkamo (Vapalahti *et al.*, 1992), Samara_49/CG/2005, Samara_94/CG/2005 (Seto *et al.*, 2011) and DTK/Ufa-97 (Abu Daude *et al.*, 2008) were propagated in Vero E6 cells. The culture fluid was collected as virus stock and stored at −80 °C until use.

Growth curve of MRK101 cells. Approximately 1.0×10^5 MRK101 cells were seeded into wells of a six-well plate, collected at 24 h intervals for 7 days by treatment with 0.167 % trypsin, and counted after staining with 0.2 % Trypan blue (Gibco). The doubling time was calculated from the growth curve.

Mitochondrial DNA (mtDNA) analysis. The mtDNA of MRK101 cells was extracted using the mtDNA extractor CT kit (Wako). Amplification and sequencing of the cytochrome *b* (*cytb*) gene was performed with universal primers (L14724 and H15915) (Irwin *et al.*, 1991). The GenBank accession no. for the *cytb* gene sequence of MRK101 cells is AB712373.

Focus-forming assay. Virus titres in culture fluid were determined with a focus-forming assay. The culture fluid of hantavirus-infected cells was serially diluted in DMEM and then inoculated onto confluent MRK101 or Vero E6 cells grown in 96-well plates. After 1 h of incubation at 37 °C, the mixture was removed and the cells were overlaid with EMEM containing 1.5 % carboxymethyl cellulose (Wako). After incubation at 37 °C for 6 days, the overlay medium was removed and the cells were washed with PBS. The cells were then fixed with methanol for 15 min at room temperature. The viral foci of PUUV and HOKV were stained with PUUV-infected hamster serum (1 : 200) (Sanada *et al.*, 2011) and Alexa Fluor 488-conjugated anti-hamster IgG (1 : 1000; Invitrogen). The viral foci of HTNV, SEOV and AMRV were stained with the mouse mAb E5/G6 (0.5 μg ml^{-1}) (Yoshimatsu *et al.*, 1996) and an Alexa Fluor 555-conjugated anti-mouse IgG (1 : 1000; Invitrogen). Stained foci were counted under a fluorescence microscope.

Analysis of hantavirus propagation in MRK101 cells and Vero E6 cells. Approximately 1.0×10^6 MRK101 and Vero E6 cells grown in wells of a six-well plate were infected with the PUUV Sotkamo strain, Samara_94/CG/2005 strain, HTNV 76-118 strain, SEOV SR-11 strain, or HOKV Kitahiyama128 strain at 0.001 m.o.i.. Culture fluids and infected cells were collected at 1, 3, 5, 7, 10 and 14 days p.i. The culture medium was changed at 7 days p.i. The collected fluids were subjected to focus-forming assays, and the presence of viral antigens in infected cells was evaluated by Western blotting.

Western blot analysis. Infected cells were diluted in SDS sample buffer, subjected to SDS-PAGE, and then transferred to a PVDF membrane. The membrane was blocked with Block Ace (Dai Nippon Pharmaceutical) at 37 °C for 1 h. The antibody reaction was performed overnight at 4 °C with the mouse mAb E5/G6 to the viral N protein (0.5 μg ml^{-1}) (Yoshimatsu *et al.*, 1996). After washing, the membrane was incubated with a 1 : 5000 dilution of peroxidase-conjugated anti-mouse IgG (Zymed) at 37 °C for 1 h. Protein detection was performed using ECL Detection Reagents (GE Healthcare). A peroxidase-conjugated anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology) was used to detect GAPDH as a loading control.

Virus isolation. Virus isolation was performed using a modified version of a method described previously (Seto *et al.*, 2011). Briefly, lung tissues from an HOKV-infected grey red-backed vole were inoculated into suckling Syrian hamsters intracerebrally. At 15 days p.i., lung and kidney samples were collected from the inoculated animals. The supernatants of lung and kidney homogenates were inoculated into MRK or Vero E6 cells. Medium was changed every week and cells were subcultured at 14 day intervals. At subculture, a proportion of cells were collected and the hantavirus genome was detected by RT-PCR. Hantaviral antigens were detected using an IFA. Supernatants were also collected and the presence of infectious virus evaluated with a focus-forming assay.

RT-PCR. Total RNA was extracted from HOKV-infected cells using ISOGEN (Nippon Gene) and reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen) with random primers (Invitrogen), according to the manufacturer's protocol. Partial S genome segments

were amplified using the forward primer HokkaidoS172Fw (5′-CTGCAAGCACGGCAACAAACAGTGTCAGCA-3′), the reverse primer HokkaidoS894Rv (5′-GTCGGGGACATGATTCTTATCAAGCACATC-3′), and the Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) for virus RNA detection.

IFA. HOKV-infected cells were collected by trypsinization and spotted onto 24-well slides. After incubation for 4 h, the cells were fixed with cold acetone for 20 min and air-dried. Hamster sera infected with PUUV Sotkamo strain were diluted to 1 : 32 and spotted onto the slide. After incubation at 37 °C for 1 h, the slides were washed three times with PBS. Alexa Fluor 488-conjugated anti-hamster IgG (Invitrogen) diluted 1 : 1000 was spotted on the slides and incubated at 37 °C for 1 h. Scattered granular fluorescence in the cytoplasm was considered a positive reaction. Nuclei were also stained with SlowFade Gold antifade reagent with DAPI (Invitrogen).

Sequencing of the hantavirus genome segments and phylogenetic analysis. The S, M and L segments of the newly isolated HOKV strain were amplified using specific primers. The amplified PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and then sequenced directly using the BigDye Terminator v3.1 Cycle Sequencing kit and ABI 3130 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. The S, M and L segment sequences of the isolated HOKV strain Kitahiyama128 were deposited in GenBank under accession numbers AB675463, AB676848 and AB712372, respectively.

The genomic sequences from the isolated HOKV and those of other hantaviruses were aligned and compared using CLUSTAL W, and phylogenetic analyses were conducted using MEGA5 (Tamura *et al.*, 2011). Phylogenetic trees were generated based on the maximum-likelihood method with 1000 bootstrap replicates.

For comparison, the sequences of hantaviruses were obtained from GenBank. The hantavirus sequences used in this study were HOKV Kamiiso-8Cr-95 (S segment, accession no. AB010730) and Tobetsu-60Cr-93 (S, AB010731); PUUV strains, CG1820 (S, M32750; M, M29979; L, M63194), Samara_49/CG/2005 (S, AB433843; M, AB433850; L, AB574183), Samara_94/CG/2005 (S, AB433845; M, AB433852; L, AB574184), Sotkamo (S, NC_005224; M, NC_005223; L, Z66548), DTK/Ufa-97 (S, AB297665; M, AB297666; L, AB297667), Kazan (S, Z84204; M; Z84205; L, EF405801) and Vranica (S, U14137; M, U14136); Muju virus (MUJV) strain 96-1 (S, DQ138133), 04-4 (M, EF198413); Topografov virus (TOPV) strain Ls136V (S, AJ011646; M, AJ011647); Khabarovsk virus (KBRV) strain MF-43(S, U35255; M, AJ011648); TULV strain 5302v (S, NC_005227; M, NC_005228; L, NC_005226); PHV strain PH-1 (S, Z49098; M, X55129; L, EF646763); SNV strain NMR11 (S, L37904; M, L37903; L, L37902); HTNV strain 76-118 (S, NC_005218; M, NC_005219; L, NC_005222); AMRV strains Khekhtsir/AP209/2005 (S, AB620028; M, AB620029; L, AB620030) and H5 (S, AB127996; M, AB127993); and SEOV strain 80-39 (S, NC_005236; M, NC_005237; L, NC_005238).

Statistical analyses. Student's *t*-test was used to conduct statistical analyses of the data.

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Development of a Diagnostic Method Applicable to Various Serotypes of Hantavirus Infection in Rodents

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ABSTRACT. Antigenic diversity among different hantaviruses requires a variety of reagents for diagnosis of hantavirus infection. To develop a diagnostic method applicable to various hantavirus infections with a single set of reagents, we developed an enzyme-linked immunosorbent assay (ELISA) using recombinant nucleocapsid proteins of three hantaviruses, Amur, Hokkaido, and Sin Nombre viruses. This novel cocktail antigen-based ELISA enabled detection of antibodies against Hantaan, Seoul, Amur, Puumala, and Sin Nombre viruses in immunized laboratory animals. In wild rodent species, including *Apodemus*, *Rattus*, and *Myodes*, our ELISA detected antibodies against hantaviruses with high sensitivity and specificity. These data suggest that our novel diagnostic ELISA is a useful tool for screening hantavirus infections and could be effectively utilized for serological surveillance and quarantine purposes.
KEY WORDS: diagnosis, ELISA, hantavirus, rodents.

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Hantaviruses are members of the family *Bunyaviridae* and possess a three-segmented genome consisting of small (S), medium (M), and large (L) segments that encode nucleocapsid protein (N), envelope glycoprotein (Gn and Gc), and RNA-dependent RNA polymerase, respectively [22]. More than 40 hantaviruses have been reported from different parts of the world in association with a variety of rodent and Soricomorpha species [9]. Hantaviruses cause two severe human diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) [9]. HFRS occurs mainly in Asia and Europe, with 150,000 to 200,000 cases annually [9, 19], and its case fatality rate is 0.1–15% [10, 19]. Hantaan virus (HTNV), Seoul virus (SEOV), Amur virus (AMRV), Dobrava-Belgrade virus (DOBV), Saaremaa virus (SAAV), and Puumala virus (PUUV) have been identified as etiologic agents of HFRS, and they are each carried by a specific rodent species: striped field mouse (*Apodemus agrarius*), Norway rat (*Rattus norvegicus*), Korean field mouse (*A. peninsulae*), yellow necked mouse (*A. flavicollis*), and bank vole (*Myodes glareolus*), respectively [9]. Sin Nombre virus (SNV), Laguna Negra virus (LNV), and Andes virus (ANDV) are considered to be the major pathogens of HCPS [4, 9]. The case fatality rate of HCPS is as high as 40% [9]. The transmission of hantavirus to humans is believed to occur by inhalation of aerosolized rodent excreta [31]. Vaccines for HFRS are only available in China and Korea, and there is no vaccine for HCPS in any country [24]. In addition, there are no antiviral drugs that can clear hantavirus infection [19]. Therefore, serological survey of wild rodents is one of the important preventive measures against human hantaviral infection.

The diagnosis of hantaviral infection in wild rodents is usually determined by indirect immunofluorescence antibody test (IFA), based on infected cells, or enzyme-linked immunosorbent assay (ELISA), based on either native or recombinant N antigen [29]. Although N protein is highly cross-reactive between related hantaviruses, there are significant antigenic differences among hantavirus N proteins [5]. Thus, for diagnostic methods using N protein, it is necessary to prepare a large number of reagents, including a variety of hantaviral N proteins and optimal secondary antibodies dependent on the animal species of interest. For these reasons, screening for hantavirus infection in various species is complicated, and antibody detection in animal species in which hantavirus infection has not been reported is especially difficult.

There are three groups of rodent-borne hantaviruses, distinguished by their host range (Murinae-, Arvicolinae-, and Sigmodontinae- or Neotominae-associated viruses [8]), and viruses in each group have cognate antigenic characteristics

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[5]. Here, to develop a simple diagnostic method applicable to multiple serotypes of hantavirus infection using a single set of reagents, we developed a new ELISA test that uses a cocktail of antigens from recombinant N proteins of three different hantaviruses.

The hantaviruses used in this study were propagated in Vero E6 cells, as described previously [1]. Immune mouse, rat, hamster, and rabbit sera specific to each hantavirus were prepared by previously described methods [12, 18, 23]. All animal experiments were performed according to the guidelines of animal experiments at the School of Veterinary Medicine, Hokkaido University, Japan, and carried out at a biosafety level 3 animal facility.

A total of 220 serum samples were collected from wild rodents captured from 1990 to 2005. *A. agrarius* (n=46) and *A. peninsulae* (n=29) were captured in the Khabarovsk region of Russia in 2004 and 2005, respectively. *M. glareolus* (n=70) were captured in the suburbs of Samara, Russia, in 2005. *R. norvegicus* (n=28) were captured in Hokuto, Hokkaido, Japan, in 1990, and *M. rufocanus* (n=47), which are the natural host of Hokkaido virus [14], were captured in Nakagawa, Hokkaido, in 2004.

Recombinant N (rN) proteins of the Hokkaido, Amur, and Sin Nombre viruses were expressed as fusion proteins with N-utilization substance A (NusA) by cloning into the pET-43.1b (+) or pET-43.1c (+) vector (Novagen, San Diego, CA, U.S.A.), as previously described [6, 13]. A mixture of these three rNs, at 0.5 μg/m*l* each, or the NusA alone, diluted to 1.5 μg/m*l*, was coated onto 96-well plates with 50 μl per well. After overnight incubation, the coated plates were blocked with 200 μl per well of 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) at 37°C for 1 hr, followed by three washes with PBS containing 0.5% Tween 20 (PBST). Then, 50 μl of each serum sample, diluted to 1:200 in PBST, were added to the plate. Each serum sample was tested for reaction with the rN and NusA proteins. After 1 hr of incubation at 37°C, the plates were washed three times with PBST. Then, the plates were incubated with 50 μl peroxidase-conjugated protein G, diluted to 1:4,000 in PBST, at 37°C for 1 hr. After washing, 100 μl *o*-phenylenediamine substrate in hydrogen peroxide was added to each well, and the plates were incubated at 37°C for 30 min. The absorbance was measured at 450 nm, and the values of sample control wells (NusA) were subtracted from the values of the corresponding sample test wells (rN) to obtain the optical density (OD) value of each sample.

All serum samples were also tested for antibodies to hantavirus by IFA, as described previously [11]. For serum samples from *A. agrarius*, *A. peninsulae*, *R. norvegicus*, and *Myodes* rodents, cells infected with HTNV 76–118 strain, AMRV H5 strain, SEOV SR-11 strain, and PUUV Sotkamo strain were used as antigens, respectively. Secondary antibodies or reagents included AlexaFluor 488-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, U.S.A.) for *Apodemus* sera, fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG (MP Biomedicals Cappel, Solon, OH, U.S.A.) for *Rattus* sera, and AlexaFluor 488-conjugated protein G (Invitrogen) for *Myodes* sera. Serum samples for which the IFA titer was ≥1:16 were considered IFA-positive.

To investigate whether our ELISA method using a mixture of three hantavirus rNs would detect hantavirus-specific antibodies from various animals, we examined immune sera from laboratory animals. Figure 1A shows the results of an experiment using 2-fold dilutions of anti-hantavirus or uninfected mouse sera. Mixed antigens reacted with sera from mice immune to HTNV (76–118 strain and Bao14 strain), AMRV (B78 strain), SEOV (SR-11 strain), and PUUV (Sotkamo strain) in a dose-dependent manner. Antibodies against SEOV and PUUV were detected in rat and hamster sera (Fig. 1B), and antibodies against SNV rN were also detected in rabbit serum in a dose-dependent manner (Fig. 1C). In contrast, uninfected sera from mice, rats, hamsters, and rabbits had no reaction to the cocktail antigen (Fig. 1A-C).

To apply the cocktail antigen-based ELISA to screening of wild rodents for hantavirus infection, we examined serum samples from various wild rodents obtained from Japan and Russia. A total of 220 serum samples were tested by ELISA and IFA to compare the sensitivities and specificities for the detection of hantavirus-specific antibodies. The cutoff values of the ELISA for each rodent species were defined as the mean absorbance value of the IFA-negative rodent samples plus three times the standard deviation. The mean OD value of IFA-negative sera of each species was quite low, ranging from 0.017 to 0.076 (Table 1), and the cutoff values for *A. agrarius*, *A. peninsulae*, *R. norvegicus*, *M. glareolus*, and *M. rufocanus* were 0.157, 0.056, 0.073, 0.066, and 0.097, respectively. As shown in Fig. 2, the OD values of most IFA-positive serum samples were higher than the respective cutoff value, and most IFA-negative sera were below the respective cutoff value. Only 2 (one *A. peninsulae* and one *R. norvegicus*) of 220 sera resulted in a false-negative reading by ELISA, and 2 sera (one *R. norvegicus* and one *M. glareolus*) resulted in a false-positive reading. In the false-negative serum of *A. peninsulae* and the false-positive serum of *M. glareolus*, the results of IFA corresponded to the result of neutralization test (data not shown). Due to insufficient amount of sera, the neutralization test was not performed in the false-negative and false-positive sera of *R. norvegicus*. Comparing the results of the ELISA and IFA, the sensitivities of the ELISA detection method in *A. agrarius*, *A. peninsulae*, *R. norvegicus*, *M. glarelus*, and *M. rufocanus* were 100%, 90.0%, 90.9%, 100%, and 100%, respectively, and the specificities were 100%, 100%, 94.1%, 98.5%, and 100%, respectively (Table 2).

To date, various serotype-specific diagnostic methods have been reported to detect anti-hantavirus antibodies [3, 20, 32]. However, because diagnostic methods for detecting antibodies to hantaviruses require optimal antigens and the corresponding secondary antibodies or reagents, there is no report of a diagnostic method applicable to various hantavirus infections. In the present study, we developed a diagnostic method that can identify various hantavirus infections using a single set of reagents.

AMRV, HOKV, and SNV N protein antigens were chosen to detect antibodies against Murinae-, Arvicolinae-, and Sigmodontinae- or Neotominae-associated viruses, re-

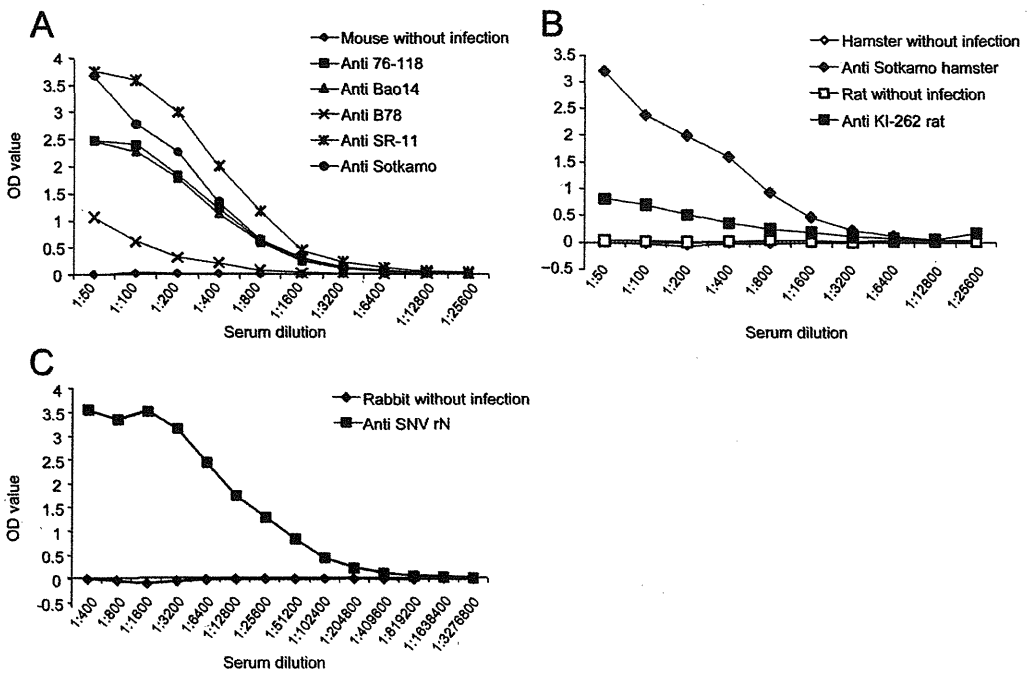

Fig. 1. Antibody detection in sera from immunized (A) mice, (B) rats, hamsters, and (C) rabbits by a novel cocktail antigen-based ELISA.

Table 1. Antibody responses of IFA-negative wild animals to our cocktail antigen-based ELISA

Rodent species	Virus	No. of samples	Mean OD value ± SD
Apodemus agrarius	HTNV	41	0.076 ± 0.027
Apodemus peninsulae	AMRV	19	0.023 ± 0.011
Rattus norvegicus	SEOV	17	0.037 ± 0.015
Myodes glareolus	PUUV	65	0.020 ± 0.015
Myodes rufocanus	HOKV	42	0.017 ± 0.026

Table 2. Detection of hantavirus-specific antibodies from various rodents

Rodent species	Virus	No. of samples					Sensitivity of ELISA	Specificity of ELISA
		Total	IFA		ELISA			
			Positive	Negative	Positive	Negative		
Apodemus agrarius	HTNV	46	5	41	5	41	100.0%	100.0%
Apodemus peninsulae	AMRV	29	10	19	9	20	90.0%	100.0%
Rattus norvegicus	SEOV	28	11	17	11	17	90.9%	94.1%
Myodes glareolus	PUUV	70	5	65	6	64	100.0%	98.5%
Myodes rufocanus	HOKV	47	5	42	5	42	100.0%	100.0%

spectively. Antibodies against Murinae-associated (HTNV, SEOV, and AMRV) and Arvicolinae-associated (PUUV and HOKV) hantaviruses were detected from infected or immunized animals in a dose-dependent manner and with high sensitivity and specificity in various wild rodent species. Antibodies against Sigmodontinae-associated (SNV) hantaviruses were also detected in immunized animals. Sera from hantavirus-uninfected wild rodents had no reaction to the cocktail antigen. These data suggest that our novel cocktail antigen-based ELISA was able to detect antibodies against hantaviruses in various rodent species.

The IFA is one of the most generally used assay for serological diagnosis of hantavirus infection [29], and the IFA result correlates well with the neutralization test result [12,

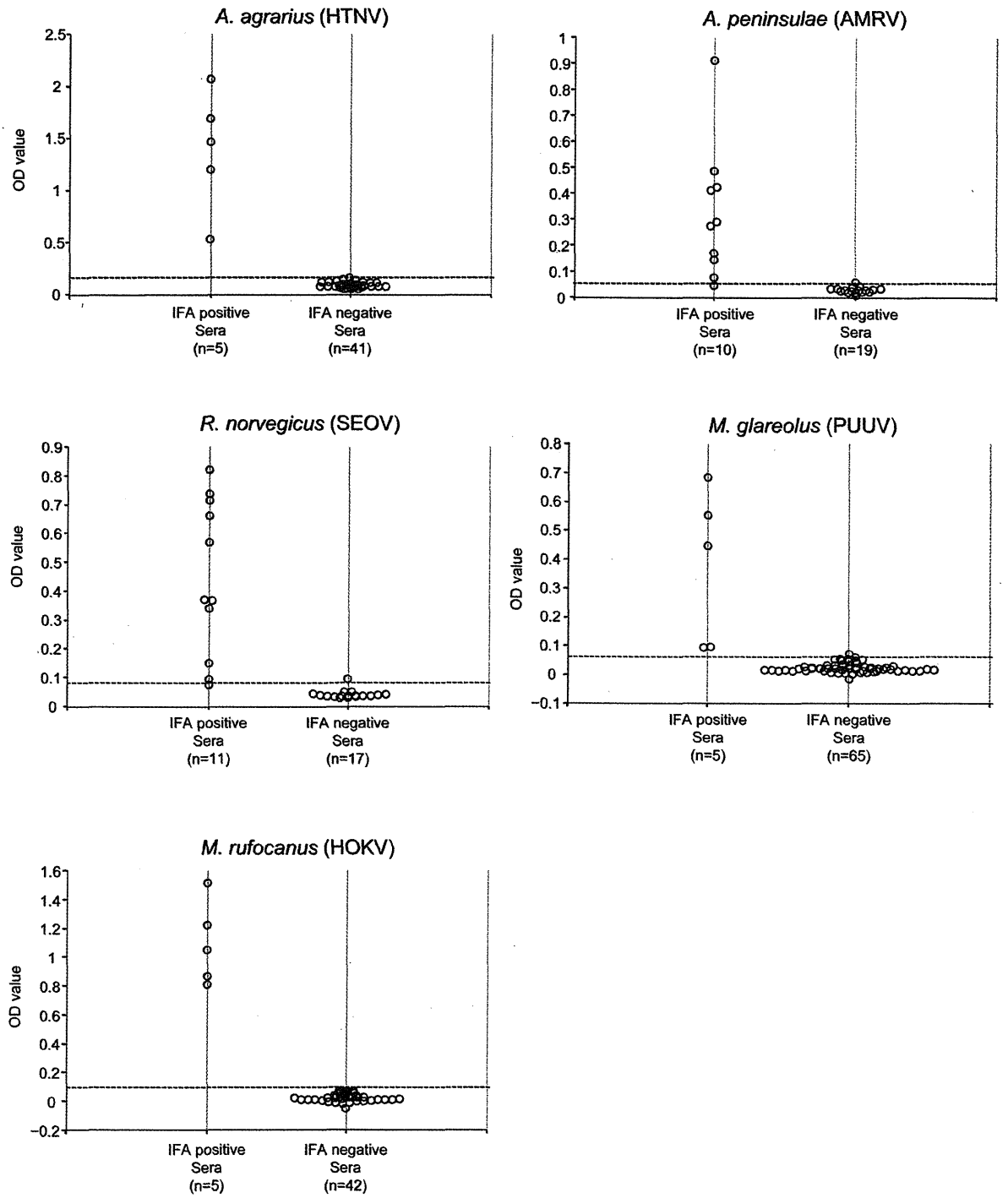

Fig. 2. Antibody detection in sera from various wild rodents by our cocktail antigen-based ELISA and by IFA. Horizontal broken lines indicate cutoff values.

27, 28]. Compared with IFA, antibody detection by ELISA in wild rodents demonstrated high sensitivity and specificity, with>90% of each for all species tested, except for 2 false-positive cases and 2 false-negative cases including one serum sample from *R. norvegicus* and one from *A. peninsulae*. In a previous study, protein G showed low reactivity

against sera from some *Apodemus* and *Rattus* rodents [17]. Our false-negative samples might be due to their reactivity with protein G. Further studies on secondary antibodies and reagents are required to improve the sensitivity of our assay.

Although it is generally believed that hantaviruses are carried by specific rodent or Soricomorpha species, there are several reports of a spillover of hantavirus from primary host animal species to other species [7, 16, 30]. Using serotype-specific diagnostic methods, it may be difficult to detect spillover of hantaviruses, because of differences in antigenicity. Because our cocktail antigen-based ELISA method can be applied to various hantavirus infections, it may be suitable for detecting such spillover.

Although there has been no report of an association between Soricomorpha-borne hantavirus and human illness, a variety of novel hantaviruses in shrews have been reported [2, 15, 25, 26]. To investigate whether the cocktail antigen-based ELISA can detect Soricomorpha-borne hantavirus infection, further studies in shrews are needed. Because antigenicity between rodent- and Soricomorpha-borne hantaviruses is quite different [21], the inclusion of antigens derived from Soricomorpha-borne hantavirus might be required in future versions of our ELISA method.

In conclusion, our novel cocktail antigen-based ELISA showed high sensitivity and specificity to various hantavirus infections and quite low reactivity to hantavirus-uninfected animal sera. Therefore, this ELISA is a useful tool for mass screening of a large variety of rodent samples as well as for serological surveillance and quarantine purposes.

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Short communication

Ecology of hantaviruses in Mexico: Genetic identification of rodent host species and spillover infection

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ABSTRACT

In our recent epidemiological survey conducted in Mexico for hantavirus infection, we identified three distinct viruses circulating in Mexican wild rodents, namely Montano virus (MTNV), Huitzilac virus (HUIV), and Carrizal virus (CARV). To gain a detailed understanding of hantavirus epidemiology and its associated hosts, 410 rodents were captured at eight collecting points in Morelos and Guerrero, Mexico, and examined for hantavirus seroprevalence, the presence of viral RNA, and rodent host species identification using cytochrome *b* gene sequences. Of the 32 species captured, seven species were positive for hantavirus: *Peromyscus beatae* (31/127; 24.4%), *Reithrodontomys sumichrasti* (6/15; 40%), *Reithrodontomys megalotis* (2/25; 8%), *Peromyscus aztecus evides* (1/1; 100%), *Peromyscus megalops* (1/41; 2.4%), *Megadontomys thomasi* (1/9; 11.1%), and *Neotoma picta* (1/6; 16.7%), with an overall prevalence of 10.5%; virus genome persisted in the majority of seropositive rodents. Nucleotide sequence and phylogenetic analysis showed that the viruses belonged mainly to the three lineages previously identified. The data showed that MTNV and CARV were primarily carried by *P. beatae* and *R. sumichrasti*, respectively. In addition, the data revealed an apparent complex interaction between hantaviruses and their hosts, suggesting active transmission and/or spillover infections within sympatric rodent species.

Hantaviruses are rodent-borne viruses that belong to the genus *Hantavirus* in the *Bunyaviridae* family. The virus genome comprises three single-stranded negative-sense RNA genome segments, called large (L), medium (M), and small (S), encoding the viral polymerase, two envelope glycoproteins (Gn and Gc), and the nucleocapsid (N) protein, respectively (Schmaljohn et al., 1987). Unlike other members of the family that are carried by arthropods, members of the genus *Hantavirus* are asymptomatically carried by persistently infected rodents (Okumura et al., 2007).

Humans are believed to acquire infection by inhalation of aerosols contaminated with infected rodent urine, feces, or saliva (Zeitz et al., 1995) and by direct contact with infected rodents. Although rodents or rodent excreta appear to be the primary source of infection for humans, evidence also points to person-to-person transmission (Martinez et al., 2005; Padula et al., 1998). Once the virus is acquired, humans may develop either hantavirus pulmonary syndrome (HPS) in North and South America, or hemorrhagic fever with renal syndrome (HFRS), which is mainly found on the Eurasian continent.

European and Asian (i.e., Old World) hantaviruses have been studied for a relatively long period of time, even before the successful isolation of Hantaan virus and the subsequent identification of *Apodemus agrarius* as the principal rodent host (Lee et al., 1982). In contrast, the importance of American (i.e., New World) hantaviruses were only recognized after a HPS outbreak in 1993 in the Southwestern United States due to infection by Sin Nombre virus (SNV) carried by *Peromyscus maniculatus* (Childs et al., 1994; Hjelle et al., 1994b). Although dozens of hantaviruses carried by a wide range of rodent species have been identified in several countries in North and South America (Jonsson et al., 2010), a considerable number of these viruses have only been genetically characterized due to the difficulty of isolating hantaviruses.

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Since hantaviruses are intimately associated with specific rodent hosts with which they are believed to have coevolved, the distribution of human disease generally mirrors that of rodent hosts. Rodent species that are known to carry HPS-causing hantaviruses in North America include, among others, *P. maniculatus* (Sin Nombre virus; SNV) (Childs et al., 1994), *Peromyscus leucopus* (New York virus; NYV) (Song et al., 1994), *Sigmodon hispidus* (Black Creek Canal virus; BCCV) (Rollin et al., 1995), *Oryzomys palustris* (Bayou virus; BAYV) (Morzunov et al., 1995), and *Oligoryzomys couesi* (Catacamas virus; CATV) (Milazzo et al., 2006). Rodents in South America known to carry hantaviruses that are pathogenic to humans include *Oligoryzomys longicaudatus* (Andes virus; ANDV) (Levis et al., 1998), *Calomys laucha* (Laguna Negra virus; LANV) (Johnson et al., 1997), *Oligoryzomys flavescens* (Lechiguanas virus; LECV) (Levis et al., 1998), and many others (Jonsson et al., 2010). Compared to other regions of the world, the hantaviruses of South America tend to be carried by different rodent species at the same time that those rodents carry other viruses, exhibiting a more complex virus–host relationship (Chu et al., 2008; Milazzo et al., 2006; Padula et al., 2004). Thus, information regarding hantaviruses and their hosts suggests that a vast number of rodent species are potential reservoirs for hantaviruses (Jonsson et al., 2010).

In Mexico in particular, none of the hantaviruses identified so far have been associated with cases of HPS. Hantavirus-related studies conducted in Mexico have identified numerous rodents as carriers of hantaviruses. However, the true extent of the diversity of potential rodent reservoirs is not yet known. For example, Playa de Oro virus (OROV), hosted by *Oryzomys couesi*, was identified in Colima in Southwestern Mexico (Chu et al., 2008), and EL Moro Canyon virus (ELMCV) was detected in *R. megalotis* (Rowe et al., 1995). Rodents for which only serological evidence of hantavirus infection is reported include *Reithrodontomys mexicanus* (Hjelle et al., 1995), *Reithrodontomys sumichrasti* (Suzan et al., 2001), *Peromyscus levipes* (Castro-Arellano et al., 2009), *Peromyscus hylocetes*, and *Peromyscus melanotis* (Mantooth et al., 2001).

In our recent epidemiological survey conducted in Mexico in 2006, we identified three distinct hantaviruses circulating in Mexican wild rodents, and these hantaviruses were designated Montano virus (MTNV), Huitzilac virus (HUIV), and Carrizal virus (CARV) (Kariwa et al., 2012). Therefore, a need existed for a thorough epidemiological investigation for the purposes of gaining greater insight into the extent of genetic diversity of Mexican hantaviruses and their relationships to host species. For this purpose an additional 200 rodents were collected (May 2007) and all the specimens ($n = 410$) were examined. The analysis of viral genes and rodent mitochondrial DNA (mtDNA) cytochrome *b* (cyt*b*) genes could reveal the maintenance and transmission patterns of hantaviruses in rodents, thus leading to a broader understanding of the virus–host interaction. Here, we present findings indicating that *P. beatae* is the principal carrier of MTNV while *R. sumichrasti* is the host of CARV.

Wild rodents were captured in May 2006 ($n = 210$) (Kariwa et al., 2012) and May 2007 ($n = 200$) for a survey of hantavirus infection at various locations in Morelos ($n = 72$) and Guerrero ($n = 338$), Mexico. Rodents were trapped and collected from two areas in Morelos (Tres Marias and Zacatepec) and six areas in Guerrero (Fig. 1 and Table 1). Measurements of body weight, sex, total body length, tail length, and hind foot-pad length of the rodents were made and recorded for subsequent identification of rodent species. Blood was collected by cardiac puncture under anesthesia and serum was isolated. Lung, kidney, spleen, heart, and liver tissues were collected and preserved at −80 °C prior to processing.

A Sin Nombre virus (SNV) construct encoding the N protein, kindly supplied by Dr. C.J. Peters (University of Texas, Galveston, TX, USA) was used for the preparation of antigen for screening rodent sera for hantavirus antibodies by enzyme-linked immunosorbent assay (ELISA), as earlier described (Kariwa et al., 2012). The S segment gene of the newly identified Mexican Montano virus (MTNV) was similarly amplified and cloned into a pET43.1b (+) vector (Novagen; EMD Chemicals, Darmstadt, Germany) to generate an N terminal NuSA-fused N protein. Rodent sera were screened for hantavirus antibodies by ELISA and Western blotting (WB) assays using the N protein of SNV or MTNV.

Total RNA from rodent lungs was extracted using the Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Rodent mtDNA from liver tissue was extracted using the mtDNA Extraction CT Kit (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's protocol. The cyt*b* gene of mtDNA was amplified using the following primers: forward, 5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′ and reverse, 5′-CTGGTTTACAAGACCAGAGTAAT-3′ (Kang et al., 2009). The nucleotide sequences of the amplified virus S genome and rodent mtDNA cyt*b* gene were determined by using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol.

The nucleotide sequences of the viral S segment open reading frame (ORF) and the rodent mtDNA cyt*b* genes were aligned using ATGC and Genetyx ver 8.0.0 (Genetics Corp, Tokyo, Japan) computer software. Nucleotide sequence alignment and phylogenetic analysis was conducted using MEGA 4 software (Tamura et al., 2007). The evolutionary relationship between virus and host was inferred using the neighbor-joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was calculated (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are presented in the units of the number of base substitutions per site.

We carried out an epidemiological survey in the Mexican states of Morelos and Guerrero. From the two surveys, 410 individual rodents were trapped in Morelos (72/410; 17.6%) and Guerrero (338/410; 82.4%) (Table 2). Infected rodents were detected at three of the eight collecting areas surveyed (Tres Marías, Carrizal de Bravo, and Chilpancingo), with an overall hantavirus seroprevalence of 10.5%. Carrizal de Bravo had both the highest number of rodents captured (200/410; 48.8%) and the highest hantavirus seroprevalence (40/200; 20.0%). The genera *Peromyscus* and *Reithrodontomys* were predominant among the several rodent species captured (Table 3). Species-specific seroprevalence was as follows: *P. beatae* (24.4%), *R. sumichrasti* (40%), *P. aztecus evides* (100%), *P. megalops* (2.4%), *R. megalotis* (8%), *M. thomasi* (11.1%), and *N. picta* (16.7%) (Table 3).

For genetic confirmation of rodent species identity, the cyt*b* gene from the 41 hantavirus-positive rodents (as determined by the presence of antibody and/or virus RNA) was amplified, and the nucleotide sequences were determined (Table 4). A database search of the rodent cyt*b* gene and morphological analysis of individual rodents revealed that hantavirus-positive rodents included *P. beatae* (29/41), *R. sumichrasti* (6/41), *P. aztecus evides* (1/41), *P. megalops* (1/41), *R. megalotis* (2/41), *M. thomasi* (1/41), and *N. picta* (1/41).

An examination of lung specimens for the presence of the hantavirus S segment by reverse transcription (RT)-PCR detected RNA in 9.5% (39/410) of individuals, of which 94.9% (37/39) were also seropositive for hantavirus (Table 4). Both hantavirus-specific antibodies and virus genome were detected in the majority of *P. beatae* and *R. sumichrasti* rodents. The entire S segment ORF was successfully amplified from 30/39 hantavirus RNA-positive rodents. The

Table 1
Rodent collection areas in Morelos and Guerrero states.

State, municipality, locality	Topography	Climate	Temp (°C)	Rainfall (mm)	Vegetation
Morelos, Huitzilac					
1a. Tres Marías, Preparatoria Comunitaria, 2852 m asl (above sea level) 19°03′33.8″N, 99°14′58.9″W	Steep volcanic soil	Tempered sub-humid	12.2	1200–1500	Pine, oak, cedar grasses
1b. Tres Marías, Cerro del Tezontle, 2870 m asl, 19°03′15.3″N, 19°15′13.1″W					
Morelos, Zacatepec					
2a. Campo experimental INIFAP, 608 m asl, 18°39′17.3″N, 99°12′08.8″W	Flat, livestock grazing	Sub-humid	24.3	892	Sugar cane, dry forest, thorn forest
2b. Cerro de La Tortuga, 980 m asl, 18°40′19.7″N, 99°12′47.2″W					
Guerrero, Iguala de La Independencia					
3a. Cerro de Tuxpan,5 km N Tuxpan, 1560 m asl, 18°23′36″N, 99°28′36″W	Slightly rough	Sub-humid	22–26	900–1100	Oak forest (3a) tropical dry forest (3b), scrub hawthorn (3c, 3d)
3b. Platanillo, 5.5 km NW Tuxpan, 1050 m asl, 18°23′56″N, 99°29′47″W					
3c. Tuxpan, 2 km E, 970 m asl, 18°21′03″N, 99°27′35″W					
3d. Tuxpan, 1.5 km E, 900 m asl, 18°20′58″N, 99°27′56″W					
Guerrero, Huitzuco de Los Figueroa					
4a. Tlaxmalac, 2.5 km SW, 900 m asl, 18°21′9″N, 99°26′08″W	Slightly rough	Sub-humid	22–26	900–1100	Tropical dry forest
4b. Tlaxmalac, 3.5 km SW, 900 m asl, 18°20′55″N, 99°26′22″W					
Guerrero, Eduardo Neri					
5a. Xochipala, 7.5 km SW, 1740 m asl, 17°46′32″N, 99°42′16″W	Rough	Semi-warm, sub-humid	18–22	900–1100	Oak forest (5a, 5b), tropical dry forest with columnar cactus (6a, 6b)
5b. Xochipala, 9 km SW, 1930 m asl, 17°45′26″N, 99°42′23″W					
6a. Zumpango del Río, 7.5 km NW, 880 m asl, 17°42′50″N, 99°33′17″W					
6b. Zumpango del Río, 3.5 km SW, 1170 m asl, 17°37′22″N, 99°33′00″W					
Guerrero, Leonardo Bravo					
7a. Carrizal de Bravo, 2.5 km SE, 2400 m asl, 17°36′35″N, 99°49′15″W	Slightly rough	Sub-humid	15–18	>1500	Cloud forest (7a, 7c, 7d, 7e) peach orchard (7b)
7b. Los Cajones, 2 km S Carrizal de Bravo, 2560 m asl, 17°36′13″N, 99°50′9″W					
7c. Las Truchas, 3 km SE Carrizal de Bravo, 2400 m asl, 17°35′54″N, 99°49′39″W					
7d. Carrizal de Bravo, 3 km S, 2700 m asl, 17°35′34″N, 99°50′14″W					
7e. Carrizal de Bravo, 4 km SE, 2700 m asl, 17°35′09″N, 99°49′58″W					
Guerrero, Chilpancingo de Los Bravos					
8a. Barranca La Imagen, 3 km SW Palo Blanco, 1130 m asl, 17°22′58″N, 99°28′50″W	Rugged, deep ravines	Semi-warm, sub-humid	18–22	>1500	Riparian forest
8b. Barranca Manguitos, 1.5 km SW Acahuizotla, 1000 m asl, 17°21′25″N, 99°28′51″W					

Numbers in the table correspond to the locations of survey sites shown in Fig. 1.

Table 2
Number of captured rodents and seroprevalence by municipalitie and location in Morelos and Guerrero.

	State	Municipality	Location	Rodents	Hantavirus positive	(%)
1	Morelos	Huitzilac	Tres Marías	60	2	3.3
2		Zacatepec	Zacatepec	12	0	0.0
		Subtotal		72	2	2.8
3	Guerrero	Iguala de La Independencia	Tuxpan	41	0	0.0
4		Eduardo Neri	Xochipala	12	0	0.0
5		Eduardo Neri	Zumpango del Río	9	0	0.0
6		Huitzuco de Los Figueroa	Tlaxmalac	20	0	0.0
7		Leonardo Bravo	Carrizal de Bravo	200	40	20
8		Chilpancingo	Palo Blanco	56	1	1.8
		Subtotal		338	41	12.1
	Total			410	43	10.5

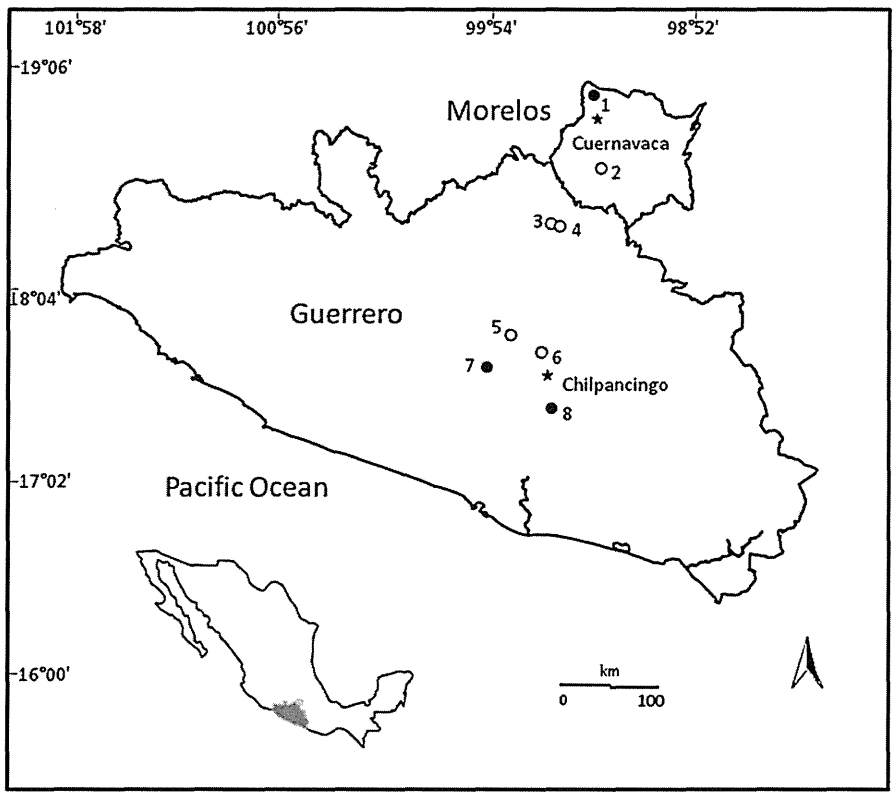

Fig. 1. The sites of wild rodent collection during 2006 and 2007 hantavirus field surveys in Morelos and Guerrero, Mexico. (1) Huitzilac, (2) Zacatepec, in Morelos, and (3) Iguala, (4) Huitzuco, (5) Eduardo Neri (Xochipala), (6) Eduardo Neri (Zumpango), (7) Leonardo Bravo, and (8) Chilpancingo in Guerrero. *, capital city of the states and collecting areas in which hantavirus-negative (○) and -positive (●) rodents were detected.

Table 3
Total number and seropositive rodents captured in various localities in Morelos and Guerrero.

Rodent	Number captured	Ab positive (%)
Morelos		
Heteromys irroratus	2	0
Microtus mexicanus	15	0
Mus musculus	12	0
Neotoma alstoni	2	0
Neotoma mexicana	1	0
Peromyscus hylocetes	13	0
Peromyscus maniculatus	2	0
Reithrodontomys megalotis	25	2(8.0)
Guerrero		
Baiomys musculus	5	0
Heteromys irroratus	13	0
Heteromys pictus	5	0
Hodomys alleni	4	0
Megadontomys thomasi	9	1(11.1)
Neotoma picta	6	1(16.7)
Handleyomys alfaroi	3	0
Oryzomys couesi	16	0
Handleyomys melanotis	2	0
Osgoodomys banderanus	36	0
Peromyscus mexicanus	10	0
Peromyscus aztecus evides	1	1(100.0)
Peromyscus beatae	127	31(24.4)
Peromyscus lepturus	5	0
Peromyscus levipes	11	0
Peromyscus megalops	41	1(2.4)
Peromyscus melanocarpus	2	0
Peromyscus melanophrys	9	0
Peromyscus spp.	14	0
Ratus rattus	1	0
Reithrodontomys spp.	1	0
Reithrodontomys sumichrasti	15	6(40.0)
Sigmodon mascotensis	1	0
Tylomys nudicaudus	1	0
Total	410	43(10.5)

proportion of hantavirus-positive male and female rodents was 88.4% and 11.6%, respectively.

To assess the evolutionary relationship between the Mexican and other known hantaviruses, we constructed phylogenetic trees based on partial S segment sequences (Figs. 2 and S1) since efforts to obtain all full S segment sequences were not successful. The analysis revealed that all of the Mexican viruses identified in this study were monophyletic with other members in the Sigmodontinae/Neotominae subfamilies. The viruses were clustered into the three major clades (with high branch node support) of MTNV (95%), CARV (99%), and HUIV (98%). Compared to known sequences of MTNV, CARV, and HUIV, the first and largest monophyletic clade of 30 taxa determined in this study was composed exclusively of MTNV that were distinct from all other previously identified hantaviruses. The analysis placed six other sequences with CARV and a single sequence with HUIV. Analysis based on the full-length S segment ORFs gave similar results, supporting the observed tree topology (Fig. S1). A comparison of the virus S segment sequence identities showed that viruses within respective groups were closely related (Table 5). The nucleotide and deduced amino acid sequence similarity within the MTN viruses ranged from 96.6% to 100% and 99.0% to 100%, respectively, while that of Carrizal viruses was 98.4% to 100% and 99% to 100%, respectively. The single sequence (96/2006) derived from *P. beatae* was more closely related to HUIV and showed a nucleotide and amino acid similarity of 99.7% and 100%, respectively.

The phylogeny based on cyt*b* gene sequence analysis of hantavirus-positive rodents showed a topology that in general reflected that of the virus gene phylogeny (Fig. 3). The analysis showed that rodents hosting MTN viruses clustered together in a single, well-supported clade (node support, 100%), indicating that these viruses were all carried by *P. beatae* with one exception: MTNV (171/2007) was found in *P. aztecus evides*. Similarly,

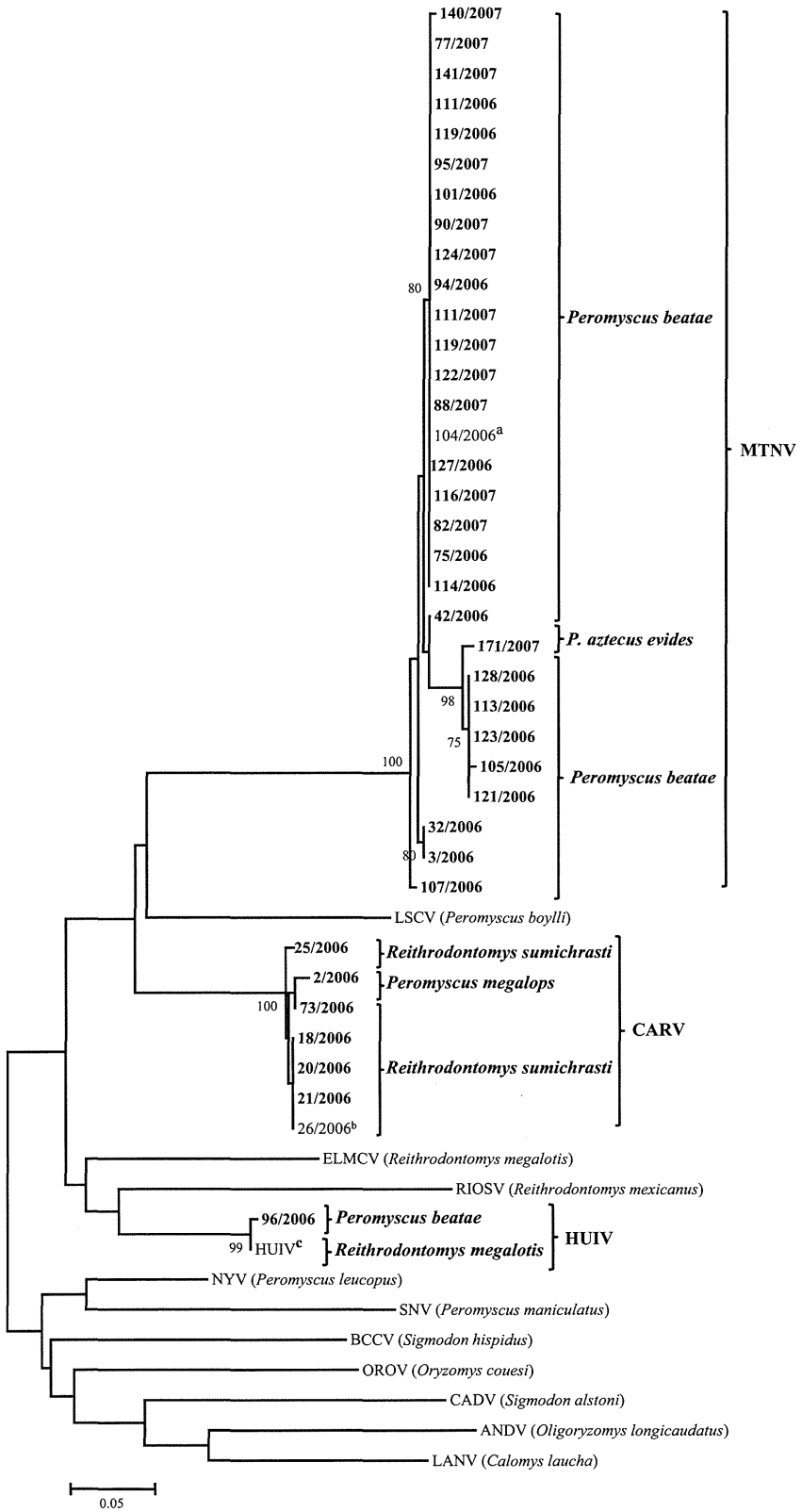

Fig. 2. A phylogenetic tree based on the partial S segment coding region was generated by the neighbor-joining (NJ) method using MEGA 4 software. The bootstrap consensus tree inferred from 1000 replicates was considered to represent the evolutionary history of the taxa ($n = 49$) analyzed. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates are not shown. In total, 312 positions were included in the final data set. The scale bar indicates the evolutionary distance of 0.05 substitutions per site. Sequences of Mexican hantavirus strains used in this study are shown in bold. Other GenBank database sequences used in the analysis are given in Table S2. a, b, and c were previously described as MTNV, CARV, and HUIV, respectively (Kariwa et al., 2012).

Table 4
Hantavirus infection in Mexican rodents.

Sample no.	Virus	Rodent	Collection site[a]	Sex	Ab	RNA	S segment	Cyt*b*
104/2006	**MTNV**	***P. beatae***	**7b**	**M**	–	**+**	**AB620100**	**AB618715**
3/2006	"	*P. beatae*	7a	M	+	+	AB620077	AB705157
32/2006	"	*P. beatae*	7a	M	+	+	AB620078	AB618643
42/2006	"	*P. beatae*	7a	M	+	+	AB620079	AB618723
75/2006	"	*P. beatae*	7c	M	+	+	AB620080	AB618710
77/2007	"	*P. beatae*	7e	M	+	+	AB703008	AB702996
82/2007	"	*P. beatae*	7e	M	+	+	AB703009	AB702997
88/2007	"	*P. beatae*	7e	M	+	+	AB703010	AB702998
90/2007	"	*P. beatae*	7e	M	+	+	AB703011	AB702999
94/2006	"	*P. beatae*	7b	M	+	+	AB620082	AB618712
95/2007	"	*P. beatae*	7d	M	+	+	AB703012	AB703000
101/2006	"	*P. beatae*	7b	M	+	+	AB620081	AB618714
105/2006	"	*P. beatae*	7b	F	+	+	AB620083	AB618644
107/2006	"	*P. beatae*	7b	M	+	+	AB620084	AB618716
111/2006	"	*P. beatae*	7b	M	+	+	AB620085	AB618717
111/2007	"	*P. beatae*	7d	M	+	+	AB703013	AB703002
113/2006	"	*P. beatae*	7b	M	+	+	AB620086	AB618718
114/2006	"	*P. beatae*	7b	M	+	+	AB620087	AB618719
116/2007	"	*P. beatae*	7d	M	+	+	AB703014	AB703003
119/2006	"	*P. beatae*[b]	7b	M	+	+	AB620088	AB705158
119/2007	"	*P. beatae*	7d	M	+	+	AB703015	ND
121/2006	"	*P. beatae*	7b	M	+	+	AB620089	AB618708
122/2007	"	*P. beatae*	7d	M	+	+	AB703016	AB703004
123/2006	"	*P. beatae*	7b	M	+	+	AB620090	AB618720
124/2007	"	*P. beatae*[b]	7d	M	+	+	AB703017	ND
127/2006	"	*P. beatae*	7b	F	+	+	AB620091	AB618721
128/2006	"	*P. beatae*	7b	F	+	+	AB620092	AB618722
140/2007	"	*P. beatae*	7d	M	+	+	AB703018	AB703005
141/2007	"	*P. beatae*	7d	M	+	+	AB703019	AB703006
171/2007	"	*P. aztecus evides*	8a	M	+	+	AB703020	AB703007
26/2006	**CARV**	***R. sumichrasti***	**7a**	**M**	**+**	**+**	**AB620103**	**AB618727**
2/2006	"	*P. megalops*	7a	M	–	+	AB620093	AB618709
18/2006	"	*R. sumichrasti*	7a	M	+	+	AB620094	AB618730
20/2006	"	*R. sumichrasti*	7a	M	+	+	AB620095	AB618731
21/2006	"	*R. sumichrasti*	7a	M	+	+	AB620096	AB618732
25/2006	"	*R. sumichrasti*	7a	F	+	+	AB620097	AB618726
73/2006	"	*R. sumichrasti*	7c	M	+	+	AB620098	AB618729
200/2006	**HUIV**	***R. megalotis***	**1a**	**M**	**+**	**+**	**AB620106**	**AB618725**
96/2006	"	*P. beatae*	7b	M	+	+	AB620099	AB618713
27/2006	ND	*N. picta*	**7a**	M	+	–	–	AB618728
92/2006	ND	*M. thomasi*	7c	M	+	–	–	AB618711
96/2007	ND	*P. beatae*	7d	M	+	–	–	AB703001
153/2006	ND	*R. megalotis*	1a	F	+	–	–	AB618724
Total		43			41	39		

ND, not determined.
[a] Numbers represent the collection site of rodents as described in Fig. 1 and Table 1.
[b] Rodent species identification only by morphology.

six of the seven CARV were detected in *R. sumichrasti*, except for CARV (2/2006), which was found in *P. megalops*. In addition, HUIV (96/2006) was detected in *P. beatae* but not in *R. megalotis* (Fig. 3).

To obtain more information on Mexican hantaviruses and their rodent hosts, we conducted epidemiological surveys in Morelos and Guerrero, Mexico. An earlier investigation identified three distinct hantaviruses circulating in Mexican wild rodents (Kariwa et al., 2012), which suggested the existence of several hantaviruses in the area. In this follow-up study, genetic analysis of virus and rodent host genes strongly indicated that *P. beatae* and *R. sumichrasti* are the primary reservoirs for MTNV and CARV, respectively.

During the two surveys, 410 rodents were trapped from eight different collecting areas, with the largest number (82%) captured in Guerrero. The high trapping rate (50%) in Carrizal de Bravo, with a high seroprevalence (20.0%), suggests a high rodent density with elevated levels of infection, a common occurrence in wild rodents (Calisher et al., 2007; Niklasson et al., 1995). *P. beatae* (127/339) and *R. megalotis* (25/72) were the predominant species at Guerrero and Morelos collecting points, respectively. The detection of antibodies and virus RNA indicate that the infected rodents species play a role as host species for hantaviruses (Childs et al., 1994). Our findings also suggest that *P. beatae* and *R. sumichrasti* are the primary hosts of MTNV and CARV, respectively. Detection of virus RNA in seropositive animals also indicates persistent infection and highlights the importance of these species in the maintenance and transmission of hantaviruses (Childs et al., 1994). Our recent rodent survey results conducted elsewhere highlighted the role of male rodents in the epidemiology of hantavirus (Daud et al., 2007).

Phylogenetic analysis of the virus and rodent gene sequences demonstrated the close association of MTNV with *P. beatae* and CARV with *R. sumichrasti*. As commonly observed with hantaviruses, this strong correlation suggests a coevolutionary relationship (Hughes and Friedman, 2000). With regard to HUIV, no firm conclusions were reached due to the constraints of a small sample size with regard to hantavirus-positive rodents. Some specific observations with sample 171/2007, a MTN virus detected in the Chilpancingo area in a *P. aztecus evides* rodent, may suggest spillover or otherwise the existence of different rodent species hosting the same virus (Morzunov et al., 1998; Nemirov et al., 2002). For example, two viruses, prototype MTNV (104/2006) identified

Table 5
Percentage nucleotide and amino acid similarity of partial S segment (321 bp) of Mexican hantaviruses and other known hantaviruses.

	MTNVs									CARVs			HUIVs		Others									
	*MTNV	75/2006	82/2007	114/2006	141/2007	42/2006	32/2006	123/2006	171/2007	*CARV	2/2006	25/2006	*HUIV	96/2006	ANDV	BCCV	CADV	ELMCV	LANV	LSCV	NYV	OROV	RIOSV	SNV
***MTNV**	-	100	100	100	100	99.4	99.1	97.2	96.9	82.9	82.2	82.6	80.1	79.8	76.0	76.6	75.7	79.1	75.7	80.7	80.1	76.9	77.9	75.0
75/2006	100	-	100	100	100	99.4	99.1	97.2	96.9	82.9	82.2	82.6	80.1	79.8	76.0	76.6	75.7	79.1	75.7	80.7	80.1	76.9	77.9	75.0
82/2007	100	100	-	100	100	99.4	99.1	97.2	96.9	82.9	82.2	82.6	80.1	79.8	76.0	76.6	75.7	79.1	75.7	80.7	80.1	76.9	77.9	75.0
114/2006	100	100	100	-	100	99.4	99.1	97.2	96.9	82.9	82.2	82.6	80.1	79.8	76.0	76.6	75.7	79.1	75.7	80.7	80.1	76.9	77.9	75.0
141/2007	100	100	100	100	-	99.4	99.1	97.2	96.9	82.9	82.2	82.6	80.1	79.8	76.0	76.6	75.7	79.1	75.7	80.7	80.1	76.9	77.9	75.0
42/2006	100	100	100	100	100	-	98.4	97.8	97.5	82.9	82.2	82.6	79.4	79.1	75.4	76.6	75.7	78.5	75.1	81.3	80.1	76.9	77.3	75.6
32/2006	99.0	99.0	99.0	99.0	99.0	99.0	-	96.9	96.6	83.2	82.9	82.9	80.7	80.4	76.9	76.9	76.3	79.4	75.4	81.0	80.7	77.6	78.5	74.4
123/2006	100	100	100	100	100	100	99.0	-	99.1	81.6	81.3	81.3	80.4	80.1	75.7	77.6	75.7	78.2	74.8	80.4	79.4	77.3	76.6	75.6
171/2007	100	100	100	100	100	100	99.0	100	-	81.9	81.6	81.6	80.1	79.8	75.4	77.6	76.3	78.5	75.1	81.0	79.8	77.3	76.6	76.6
***CARV**	92.2	92.2	92.2	92.2	92.2	92.2	93.2	92.2	92.2	-	99.1	98.8	86.6	86.3	76.0	76.9	77.3	83.2	78.5	84.1	80.7	78.8	81.9	79.1
2/2006	92.2	92.2	92.2	92.2	92.2	92.2	93.2	92.2	92.2	100	-	98.4	86.3	86.0	76.3	76.6	77.6	82.6	78.2	84.1	80.4	79.1	81.6	80.0
25/2006	91.2	91.2	91.2	91.2	91.2	91.2	92.2	91.2	91.2	99.0	99.0	-	85.7	85.4	75.4	76.3	76.3	82.2	78.2	83.8	80.1	78.5	81.3	78.5
***HUIV**	91.3	91.3	91.3	91.3	91.3	91.3	92.2	91.3	91.3	96.1	96.1	95.1	-	99.7	76.6	77.9	76.3	82.9	77.3	81.0	77.6	78.2	82.6	80.0
96/2006	91.3	91.3	91.3	91.3	91.3	91.3	92.2	91.3	91.3	96.1	96.1	95.1	100	-	76.3	78.2	76.0	83.2	76.9	80.7	77.9	78.5	82.9	79.7
ANDV	81.6	81.6	81.6	81.6	81.6	81.6	82.5	81.6	81.6	85.4	85.4	85.3	84.5	84.5	-	78.8	79.8	76.9	82.2	75.4	78.5	77.6	73.8	75.9
BCCV	85.4	85.4	85.4	85.4	85.4	85.4	86.4	85.4	85.4	85.4	85.4	85.3	88.4	88.4	90.3	-	78.2	81.3	80.1	76.3	81.3	79.1	77.3	79.4
CADV	78.6	78.6	78.6	78.6	78.6	78.6	79.6	78.6	78.6	83.5	83.5	84.2	82.5	82.5	89.3	84.5	-	79.8	80.4	76.9	80.7	80.7	75.7	79.7
ELMCV	90.3	90.3	90.3	90.3	90.3	90.3	91.3	90.3	90.3	94.2	94.2	93.1	97.1	97.1	85.4	89.3	82.5	-	77.6	79.1	79.1	81.0	80.7	77.5
LANV	79.6	79.6	79.6	79.6	79.6	79.6	80.6	79.6	79.6	84.5	84.5	84.3	84.5	84.5	92.2	88.4	90.3	83.5	-	78.2	77.9	78.2	76.3	78.8
LSCV	95.1	95.1	95.1	95.1	95.1	95.1	96.1	95.1	95.1	95.1	95.1	94.1	93.2	93.2	82.5	86.4	79.6	91.3	82.5	-	78.2	80.4	80.7	79.7
NYV	85.4	85.4	85.4	85.4	85.4	85.4	86.4	85.4	85.4	85.4	85.4	86.1	85.4	85.4	89.3	90.3	87.4	85.4	87.4	87.4	-	81.0	77.9	82.5
OROV	82.5	82.5	82.5	82.5	82.5	82.5	83.5	82.5	82.5	87.4	87.4	87.3	89.3	89.3	87.4	92.2	88.4	89.3	90.3	86.4	88.4	-	80.1	79.7
RIOSV	91.3	91.3	91.3	91.3	91.3	91.3	92.2	91.3	91.3	94.2	94.2	93.1	94.2	94.2	84.5	88.4	80.6	92.2	82.5	93.2	85.4	86.4	-	80.0
SNV	91.0	91.0	91.0	91.0	91.0	91.0	90.0	91.0	91.0	88.0	88.0	88.0	89.9	89.9	89.0	92.0	87.3	90.0	86.1	89.0	92.2	90.1	86.0	-

*Prototype Mexican hantaviruses MTNV (104/2006), CARV (26/2006) and HUIV (200/2006) (Kariwa et al., 2012).

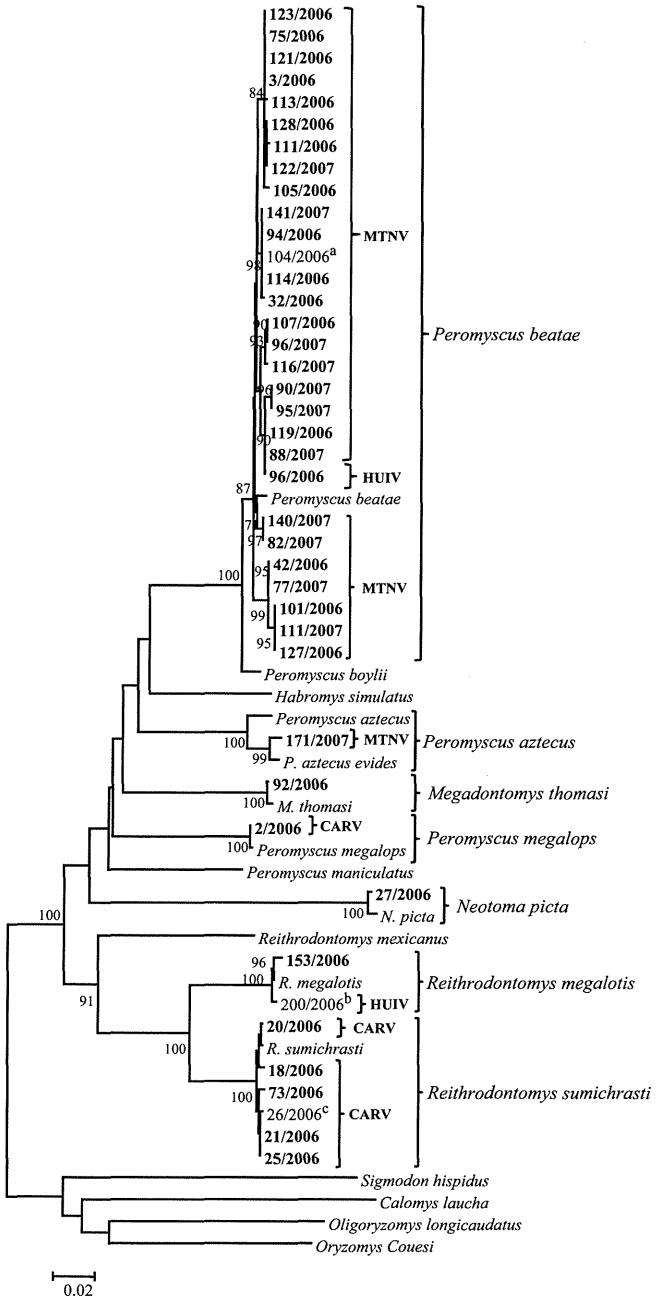

Fig. 3. A phylogenetic tree based on the partial mtDNA cyt*b* gene was generated by the neighbor-joining (NJ) method using MEGA 4 software. The bootstrap consensus tree inferred from 1000 replicates was considered to represent the evolutionary history of the taxa ($n = 57$) analyzed. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates are not shown. In total, 925 positions were included in the final data set. The scale bar indicates the evolutionary distance of 0.02 substitutions per site. Sequences of Mexican hantavirus strains used in this study are shown in bold: other GenBank database sequences used in the analysis are given in Table S2. a, b, and c were previously described as MTNV, HUIV, and CARV, respectively (Kariwa et al., 2012).

in *P. beatae* and MTNV (171/2007) detected in *P. aztecus evides*, were identified in two separate surveys, and their respective S segment ORFs had nucleotide and amino acid similarity of 96.9% and 100%, respectively, an indication that these were actually the same virus. Furthermore, the fact that these viruses were obtained from geographically distinct locations may indicate that the same virus was hosted by two different rodent species. Extensive sampling of rodents in this location would help clarify these observations.

The detection of CARV (2/2006) in *P. megalops* in the Carrizal area, where *R. sumichrasti* were the predominant rodents, suggest the possibility of spillover of this virus to *P. megalops*. In addition, lack of seroconversion suggested that this may have been a recent infection. Since HUIV (96/2006) was identified in *P. beatae*, in which MTNV was the predominant virus, it is tempting to speculate that a probable spillover event took place from *Reithrodontomys* species to *P. beatae*.

From these findings, the complex interactions of hantaviruses and their hosts have become evident. The role of these rodents in the epidemiology of Mexican hantaviruses may be extensive, and the virus–host relationship may be further complicated by obscure rodent taxonomy (Bradley et al., 2007). Previous seroprevalence studies have found other rodent species, such as *P. levipes*, *N. picta*, and *M. thomasi*, to be potential carriers of hantaviruses (Castro-Arellano et al., 2009; Mantooth et al., 2001). Additional work to detect viral RNA in these rodent species is needed, since attempts to amplify viral RNA in these rodents have been unsuccessful.

The importance of these Mexican hantaviruses as human pathogens still remains to be clarified. To date, unlike *Peromyscus*-borne viruses, *Reithrodontomys*-borne hantaviruses have not been linked to HPS in humans (Hjelle et al., 1994a, 1995). Screening for hantavirus-specific antibodies in local residents could provide data on the extent of exposure of humans to these potential pathogens.

Despite the relatively high number of infected rodents, the risk of these viruses to the local human population needs to be investigated, as no obvious correlation between rodent seroprevalence and human infection usually exists (Yates et al., 2002). Evidence obtained from this study highlights the importance of *P. beatae* and *R. sumichrasti* in the epidemiology of hantaviruses in Mexico. This study suggests the existence of an extensive relationship between Mexican rodents and hantaviruses, necessitating appropriate precautions that would minimize exposure to these rodents or their contaminated remains. Nevertheless, more studies are required to establish trends and dynamics in virus–rodent host interactions in the country. In addition, isolation of these Mexican viruses, although attempts have so far been unsuccessful, could provide opportunities for in-depth understanding of these hantaviruses.

This study provides valuable data toward understanding the epidemiology of Mexican hantaviruses and their hosts. Here, we report that *P. beatae* is the host for MTNV, and *R. sumichrasti* is the host for CARV.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2012.06.020.