likely to possess uncommon *Campylobacter* species. To assess the risk and potential source, wild birds in the same habitat and the environment where the animals live should be investigated.

Conclusion

In the present study, we investigated the prevalence of three important enteric pathogens, including *Salmonella*, *Yersinia*, and *Campylobacter* spp. in feral raccoons and masked palm civets. These results lead to the conclusion that these animals are potential reservoirs of the pathogens. The characteristics of the isolates showed that these animals probably acquired the pathogens from human activities, other wild animals, and the environment. The presence of human-associated serovars and the antimicrobial resistance of the *Salmonella* isolates revealed the effect of human activities on these animals. This represents a typical spill-over of pathogens from human activities to wildlife (Daszak et al., 2000). Meanwhile, the carrying of pathogens which are usually isolated from wildlife or from the environment (e.g. *S. enterica* subsp. *diarizonae* and *Y. pseudotuberculosis*) indicated that these animals could play an important role in the life cycles of those bacteria in their habitats. These findings are in concordance with their omnivore behaviour and their wide range of habitats from forests to urban areas (Zeveloff, 2002; Abe, 2005).

Our results revealed that raccoons and masked palm civets play an important role on the spreading of human-related pathogens. Moreover, carriage of wildlife- and environment-related pathogens in these animals showed the possibility of the transmission of these pathogens to humans and domestic animals. Raccoons and masked palm civets live near areas of human habitation and often nest in attics or in the feed stores of livestock. The enteric pathogens that we investigated can be transmitted to humans and domestic animals via feces, contaminated water and soil (Fukushima et al., 1988; Humphrey and Bygrave, 1988; Handeland et al., 2002). Thus, not only the ecological threats but also the public and animal health risks presented by these animals should be assessed in detail.

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Isolation of Hokkaido virus, genus *Hantavirus*, using a newly established cell line derived from the kidney of the grey red-backed vole (*Myodes rufocanus bedfordiae*)

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Hantaviruses belong to the family *Bunyaviridae* and are maintained in wild rodents. Although Vero E6 cells, which originate from African green monkey kidney, are used widely in hantavirus research, isolation of hantaviruses from this cell line is difficult. To develop an efficient method of propagation and isolation of hantaviruses we established a novel cell line, MRK101, derived from the kidney of the grey red-backed vole (*Myodes rufocanus bedfordiae*), the natural host of Hokkaido virus (HOKV). The MRK101 cells showed a significantly higher susceptibility to Puumala virus (PUUV) hosted by *Myodes glareolus* than Vero E6 cells. Viral nucleocapsid protein in PUUV-infected MRK101 cells was detected earlier than in Vero E6 cells, and the viral titre in the culture fluid of MRK101 cells was higher than that of Vero E6 cells during the early phase of infection. In contrast, MRK101 cells showed no susceptibility to Hantaan virus. HOKV, which has not been isolated to date, was isolated successfully using MRK101 cells. Moreover, the newly isolated HOKV was successfully propagated in MRK101, but not Vero E6, cells. Phylogenic analyses of the S (small), M (medium) and L (large) segment sequences revealed that HOKV is related most closely to PUUV, but is distinct from other hantaviruses. These data suggest that the MRK101 cell line is a useful tool for the isolation and propagation of hantaviruses. Moreover, this is (to our knowledge) the first report of hantavirus isolation in a cell line that originated from the natural host.

INTRODUCTION

Hantaviruses, members of the family *Bunyaviridae*, have a tri-segmented, negative-stranded RNA genome consisting of small (S), medium (M) and large (L) segments (Plyusnin *et al.*, 1996), which encode nucleocapsid (N) proteins, envelope glycoproteins (Gn and Gc) and an RNA-dependent RNA polymerase (RdRP), respectively (Plyusnin *et al.*, 1996). Each hantavirus is carried by a specific species in the orders Rodentia and Soricomorpha. For example, Hantaan virus (HTNV), Seoul virus (SEOV), Amur virus (AMRV) and Puumala virus (PUUV) are carried by the striped field mouse (*Apodemus agrarius*), Norway rat (*Rattus norvegicus*), Korean field mouse (*Apodemus peninsulae*) and bank vole (*Myodes glareolus*), respectively (Jonsson *et al.*, 2010). In general, hantaviruses are believed to infect persistently, and are apathogenic to their reservoir hosts (Gavrilovskaya *et al.*, 1990; Yanagihara *et al.*, 1985). In contrast, hantaviruses when transmitted to humans cause two severe disease syndromes: haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). HTNV, SEOV, AMRV, Dobrava-Belgrade virus, Saaremaa virus and PUUV have been identified as aetiological agents of HFRS. Sin Nombre virus (SNV), Laguna Negra virus and Andes virus (ANDV) are considered to be the major pathogens associated with HCPS (Bi *et al.*, 2008; Jonsson *et al.*, 2010).

Hokkaido virus (HOKV) is a hantavirus that is maintained in grey red-backed voles (*Myodes rufocanus bedfordiae*), which are distributed geographically in the northern Palaearctic, extending from northern Fennoscandia through northern Russia and the northern part of Asia (Sheftel & Henttonen, 2008). Although HOKV is related serologically to PUUV, these viruses are distinct genetically (Kariwa *et al.*, 1999). No human disease cases have been reported in association with HOKV. Although HOKV was first identified in 1995 (Kariwa

The GenBank/EMBL/DDBJ accession numbers for the S, M and L segment sequences of HOKV strain Kitahiyama128/2008 and the *cytb* gene sequence of MRK101 cells are AB675463, AB676848, AB712372 and AB712373, respectively.

et al., 1995), little is known about its characteristics due to the inability to isolate this virus.

Several cell-culture systems for the study of hantavirus infection have been reported to date (Ohsawa *et al.*, 1995; Pensiero *et al.*, 1992; Raftery *et al.*, 2002; Yanagihara & Silverman, 1990; Yanagihara *et al.*, 1984). Since it is derived from African green monkey kidney, exhibits a functional type III interferon response but not type I interferon response (Prescott *et al.*, 2010; Stoltz & Klingström, 2010), and is susceptible to hantaviruses, the Vero E6 cell line is used widely in hantavirus research, particularly for their isolation and propagation. However, hantaviruses do not grow well even in Vero E6 cells, and isolation is not always successful (Seto *et al.*, 2011). Therefore, generation of a novel cell line that shows a greater susceptibility to hantaviruses, and from which hantavirus could be isolated easily, is required.

Hantaviruses are known to be associated strongly with their specific rodent hosts (Jonsson *et al.*, 2010). In a previous report, primary kidney cells derived from the bank vole showed a high susceptibility to PUUV (Temonen *et al.*, 1993). Recently, cell lines derived from the bank vole were established and showed PUUV susceptibility (Essbauer *et al.*, 2011; Stoltz *et al.*, 2011). These data suggest that any given hantavirus may propagate efficiently in cells derived from its natural host. Herein, we describe the establishment of a novel cell line derived from a wild rodent species that is a natural host of hantavirus and the isolation of a hantavirus using this cell line.

RESULTS

Cell line derived from the grey red-backed vole

Primary cells derived from the kidney of the grey red-backed vole (*M. rufocanus bedfordiae*), the natural host of HOKV, were cultured in a flask at 37 °C in a 5 % CO_2 incubator, and the first subculture was performed after 10 days incubation. From the seventh passage, growth stabilized and the cells were then subcultured continuously with a split rate of 1 : 3 every 3–5 days. After cloning twice by limiting dilution, the cells were designated the MRK101 cell line. MRK101 cells exhibited a cobblestone morphology, which is typical of epithelial cells (Fig. 1a). The MRK cells had a doubling time of 34.7 h, and their growth plateaued by contact inhibition around 5 days after seeding (Fig. 1b). The morphological and growth characteristics of MRK101 cells did not change during the subsequent ~100 passages. The characteristics of the cells were maintained after freezing and thawing. Tests for contamination with HOKV, lymphocytic choriomeningitis virus (LCMV) and mycoplasma were negative. No micro-organisms (i.e. bacteria, fungi and yeasts) were detected, and no cytopathic effect was observed.

To confirm the origin of the MRK101 cell line, the mitochondrial cytochrome *b* (*cytb*) gene was sequenced. The *cytb* sequence of MRK101 cells was highly similar (>99 %) to those of grey red-backed voles from Hokkaido, Japan (e.g. GenBank accession no. AB031560).

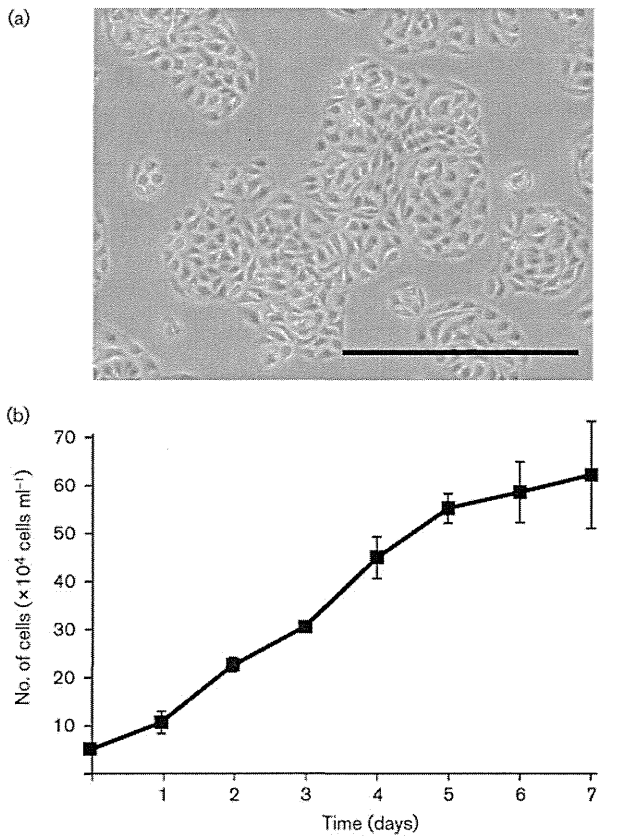

Fig. 1. Morphology and growth curve of MRK101 cells. (a) Epithelial-like morphology of MRK101 cells 72 h after seeding. Bar, 500 μm. (b) Growth curve of MRK101 cells. Data are shown as means ± SD of three independent determinations.

Susceptibility of MRK101 cells to hantaviruses

To investigate the susceptibility of MRK101 cells to hantaviruses, both MRK101 and Vero E6 cells, which are commonly used for hantavirus research, were infected with an identical infectious dose of various hantaviruses. The focus number of PUUV strains in MRK101 cells was about three times greater than those in Vero E6 cells, with the exception of the DTK/Ufa-97 strain (Fig. 2). The infectivity of the PUUV DTK/Ufa-97 strain and SEOV in MRK101 cells was identical to that in Vero E6 cells. In contrast, the susceptibility of MRK101 cells to AMRV infection was notably lower than that of Vero E6 cells. The number of AMRV foci in MRK101 cells was approximately one-twentieth of that in Vero E6 cells. No HTNV foci were observed in MRK101 cells.

Replication of hantaviruses in the MRK101 cell line

To assess the propagation of hantaviruses in MRK101 and Vero E6 cells, we evaluated the expression of hantavirus N protein in infected cells by Western blotting and

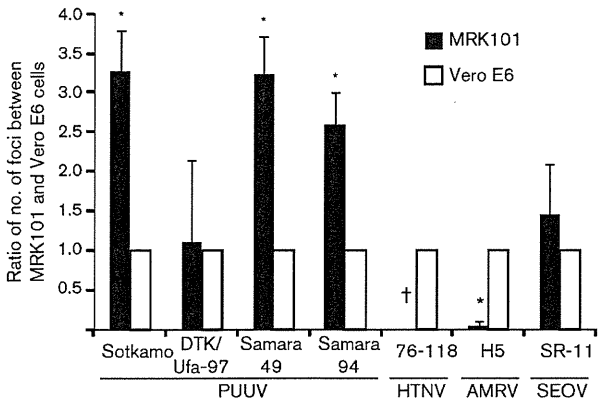

Fig. 2. Susceptibility of MRK101 and Vero E6 cells to each hantavirus. Both cell lines were infected with an identical hantavirus dose and the number of foci were compared. *Significant differences in focus number between MRK101 and Vero E6 cells ($P<0.05$); †no virus-specific foci were observed in HTNV-infected MRK101 cells.

determined the viral titre in culture fluid using a focus-forming assay. Viral N protein in MRK101 cells infected with the PUUV Sotkamo strain was initially detected at 5 days post-inoculation (p.i.) (Fig. 3a). In contrast, no PUUV antigens were detected in infected Vero E6 cells during the experimental period. Viral titres in the culture fluid of MRK101 cells were higher than that in Vero E6 cell culture fluid during the early phase of infection (Fig. 3b). At 14 days p.i., the viral titre of MRK101 cell fluid was similar to that of Vero E6 cell culture fluid. The PUUV Samara_94/CG/2005 strain showed a tendency identical to that of the PUUV Sotkamo strain (Fig. 3c, d); viral antigens in infected MRK101 cells were detected from 5 days p.i. Viral N proteins were detected in infected Vero E6 cells beginning at 10 days p.i. Viral N proteins were detected from 5 days p.i. in the culture fluid of Vero E6 cells infected with the HTNV 76-118 strain (Fig. 3e). Viral titres in the culture fluid of infected Vero E6 cells increased over time and peaked at 7 days p.i. (Fig. 3f). In contrast, neither viral antigens nor culture-fluid virus were detected in MRK101 cells infected with HTNV. In contrast to HTNV, the propagation of the SEOV SR-11 strain in MRK101 and Vero E6 cells were similar (Fig. 3g, h). Viral N proteins in both MRK101 and Vero E6 cells were detected from 5 and 7 days p.i., respectively. Viral titres in the culture fluid of both MRK101 and Vero E6 cells increased gradually and plateaued at 7 days p.i. The peak titre of culture fluid of MRK101 cells was slightly lower than that of Vero E6 cells.

Isolation of HOKV using MRK101 cells

Since MRK101 cells showed a high susceptibility to PUUV, we attempted to isolate HOKV using MRK101 cells. RT-PCR-positive lung homogenates from grey red-backed voles trapped in 2008 were inoculated into suckling Syrian hamsters for propagation of HOKV. At 15 days p.i., the lungs and kidneys of inoculated hamsters were collected and used to inoculate MRK101 and Vero E6 cells. The hantaviral genome and characteristic intracytoplasmic granular fluorescence were detected in MRK101 cells beginning at 14 days p.i. (Fig. 4a, b). Infectious virus was recovered from MRK101 cells and designated Kitahiyama128/2008 (Kitahiyama128). In contrast, virus isolation using Vero E6 cells was unsuccessful. Although viral genome was detected beginning at 14 days p.i. (Fig. 4a) and continuously detected until 84 days p.i., no virus-specific intracytoplasmic fluorescence or infectious virus was detected in Vero E6 cells until 112 days p.i.

Growth of HOKV in MRK101 cells

To assess the growth of HOKV, the HOKV Kitahiyama128 strain was inoculated into MRK101 and Vero E6 cells, and the expression of the viral N protein and the titres of progeny virus were determined. In MRK101 cells, the expression of N protein was confirmed at 14 days p.i. (Fig. 5a). The level of progeny virus increased gradually until 14 days p.i. (Fig. 5b). No cytopathic effect was observed in HOKV-infected MRK101 cells. In contrast, propagation of HOKV in Vero E6 cells was unsuccessful; expression of neither N protein nor infectious virus was detected (Fig. 5a, b).

Genetic analysis of HOKV

The full-length sequences of the S, M and L segments of the HOKV Kitahiyama128 strain were determined. The full-length S segment was 1832 nt with 42 nt 5′ and 488 nt 3′ non-coding regions and was found to encode an N protein of 433 aa. The S segment sequence of this isolated HOKV was almost identical to previously reported HOKVs (97–98.7 % nucleotide and 99.8–100 % amino acid identity) (Table 1). HOKV was the most closely related to PUUV (81.9–83.8 % nucleotide and 94.7–95.8 % amino acid identity). The complete sequence of the HOKV M segment was determined to be 3682 nt with 40 nt 5′ and 195 nt 3′ non-coding regions, and was found to encode a precursor glycoprotein of 1148 aa. The pentapeptide motif (WAASA) previously identified as the cleavage site of the glycoprotein precursor was conserved at aa 654–658. The HOKV glycoprotein contains five potential *N*-glycosylation sites, three of which are in Gn (aa 142, 357 and 409), and two are in Gc (aa 898 and 937). Of the hantavirus genome segments, the nucleotide sequence of the M segment showed the greatest variation. In terms of the M segment sequence, the most closely related virus to HOKV was PUUV, with nucleotide and amino acid identities of 78.1–79.9 % and 90.6–91.6 %, respectively. The full-length L segment comprised 6547 nt with 37 nt 5′ and 43 nt 3′ non-coding regions, and was found to code for an RdRP of 2156 aa. The pattern of genetic identities of the L segment between HOKV and other hantaviruses was similar to those of the S segment.

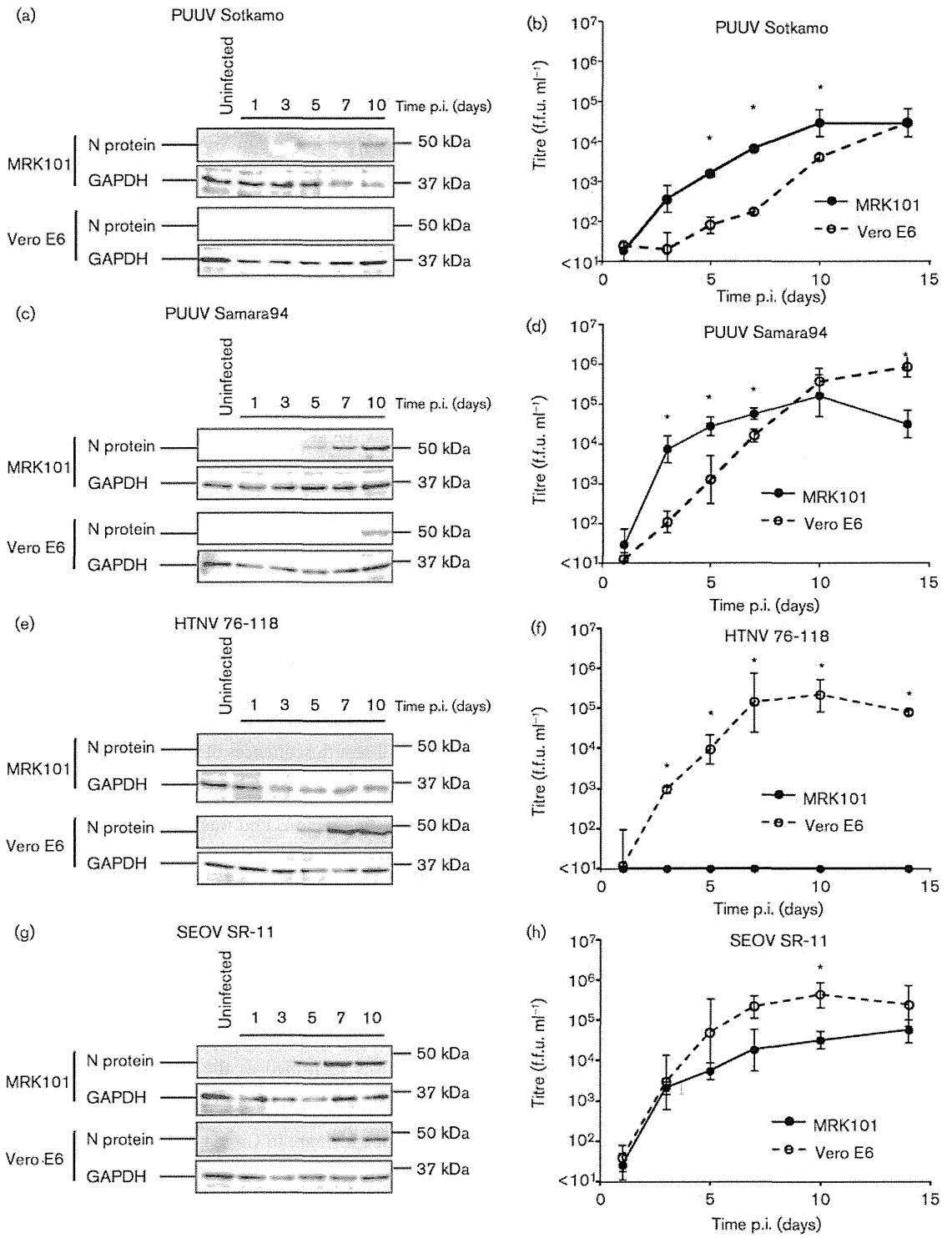

Fig. 3. Expression of hantavirus N protein in infected MRK101 and Vero E6 cells, and virus titres in culture fluid. MRK101 and Vero E6 cells were infected with the PUUV Sotkamo strain (a, b), Samara_94/CG/2005 strain (c, d), HTNV 76-118 strain (e, f) or SEOV SR-11 strain (g, h) (0.001 m.o.i.). Expression of viral protein and GAPDH in infected cells at each time point were evaluated by Western blotting (a, c, e, g). Virus titres in culture fluid, expressed as focus-forming units (f.f.u.) ml^{-1}, at each time point were determined by a focus-forming assay (b, d, f, h). The culture medium was changed at 7 days p.i. Error bars represent SD; *significant differences between MRK101 and Vero E6 cells ($P<0.05$).

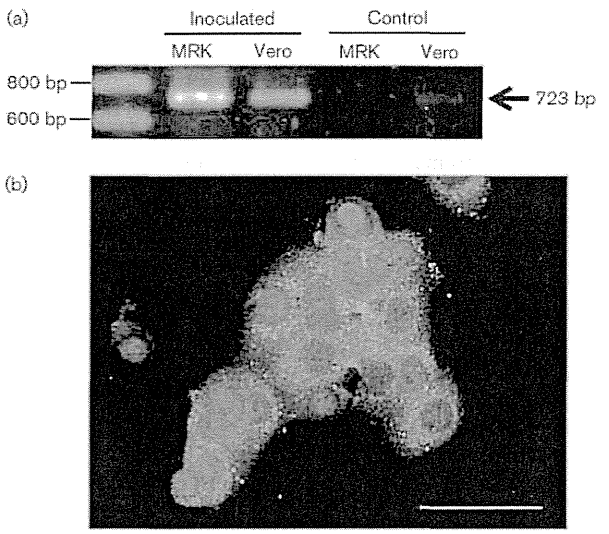

Fig. 4. Confirmation of the isolation of HOKV. (a) Detection of the HOKV genome in tissue-inoculated MRK101 and Vero E6 cells at 14 days p.i. by RT-PCR. HOKV-specific products (723 bp) were amplified from RNA extracted from inoculated MRK101 and Vero E6 cells. (b) Immunofluorescence of tissue-inoculated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

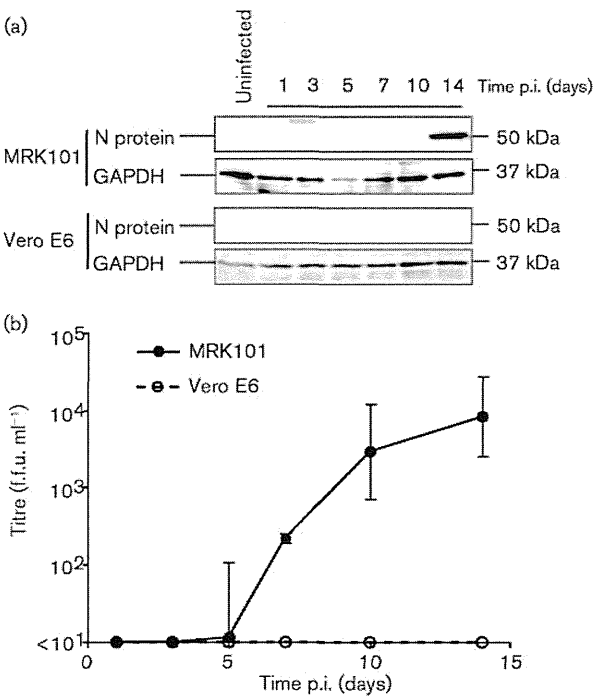

Fig. 5. MRK101 cells and Vero E6 cells infected with HOKV strain Kitahiyama128/2008. (a) Expression of viral N protein in HOKV-infected MRK101 and Vero E6 cells. (b) Virus titres in culture fluid of HOKV-infected MRK101 and Vero E6 cells. Error bars represent SD.

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

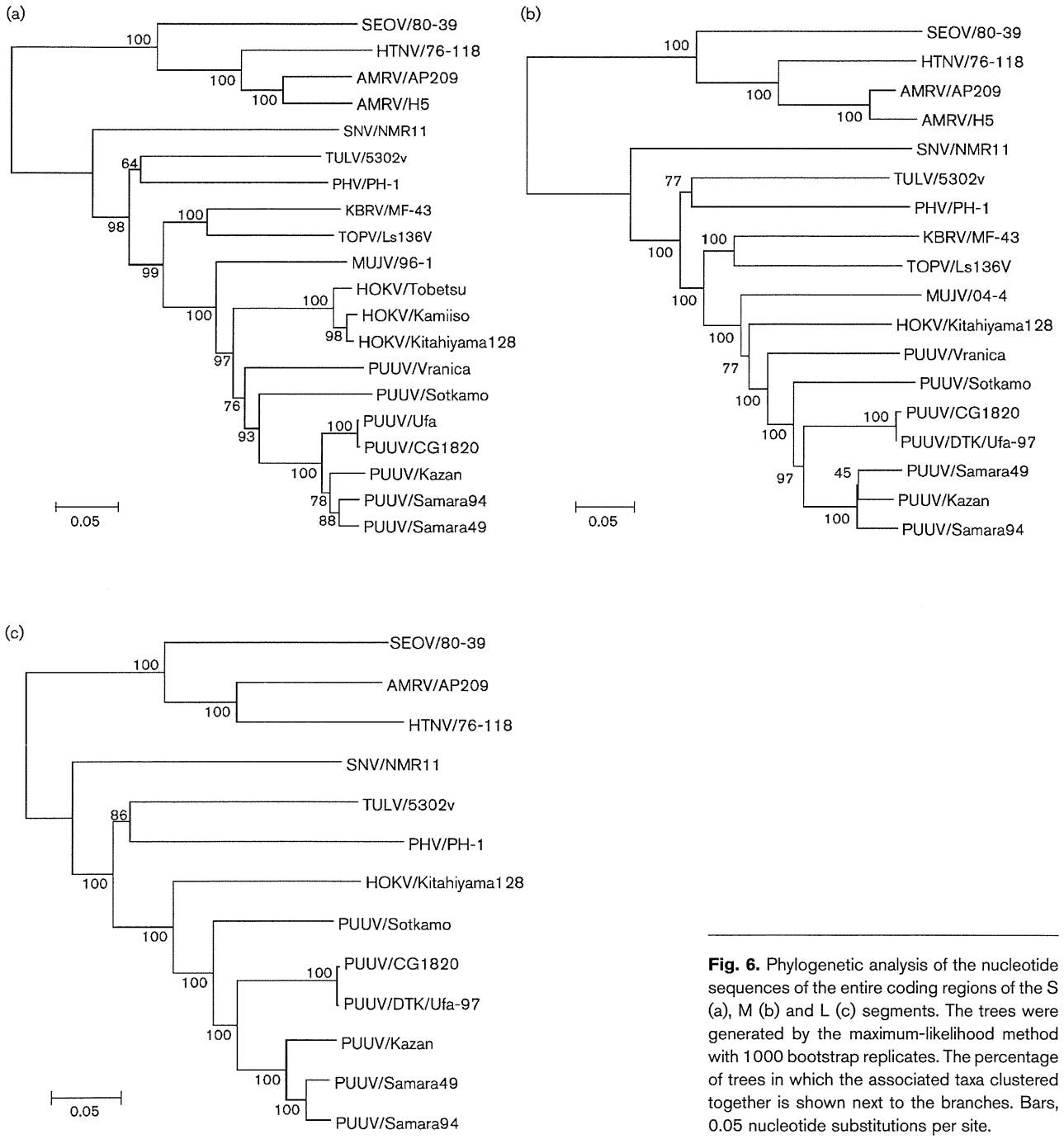

Fig. 6. Phylogenetic analysis of the nucleotide sequences of the entire coding regions of the S (a), M (b) and L (c) segments. The trees were generated by the maximum-likelihood method with 1000 bootstrap replicates. The percentage of trees in which the associated taxa clustered together is shown next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

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-

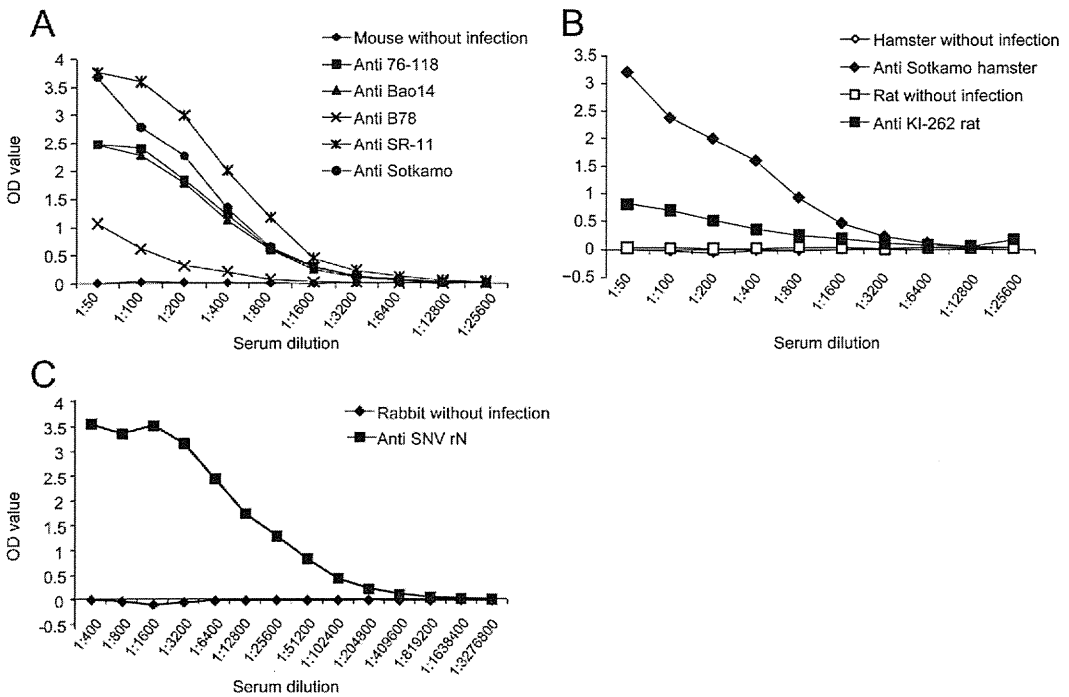

Fig. 1. Antibody detection in sera from immunized (A) mice, (B) rats, hamsters, and (C) rabbits by a novel cocktail antigen-based ELISA.

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,

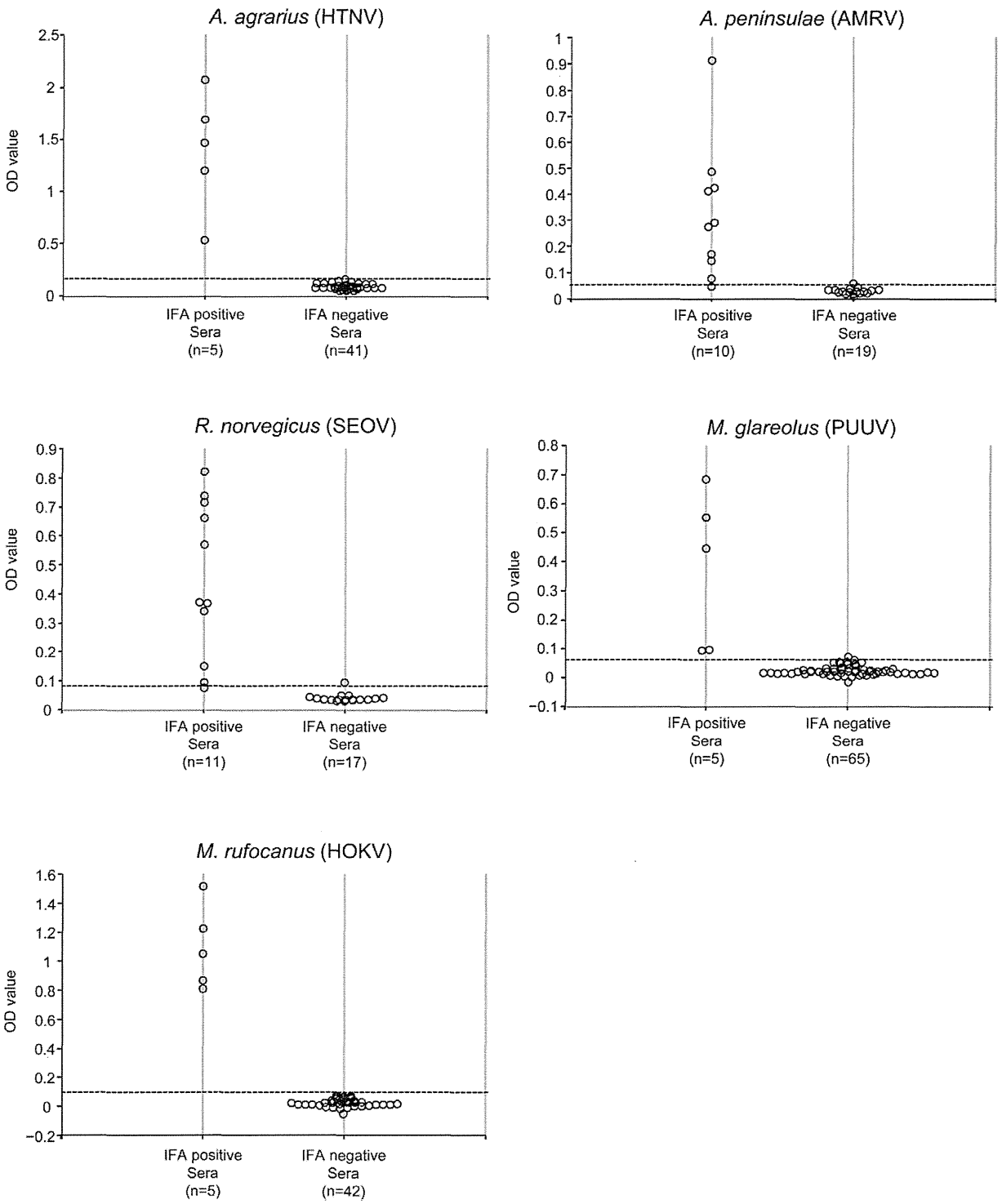

Fig. 2. Antibody detection in sera from various wild rodents by our cocktail antigen-based ELISA and by IFA. Horizontal broken lines indicate cutoff values.

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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