erties of THAIV to provide new insights into the relatedness of THAIV to other hantavirus species and confirm the classification of THAIV as a distinct hantavirus species. In addition, serological surveillance of hantavirus infection among rodents indicated the prevalence of THAIV infection mainly among giant bandicoot rats and *Rattus* species in Thailand. Finally, we present the first case of an infection with THAIV or a hantavirus antigenically more closely related to THAIV than to HTNV, SEOV, or DOBV in a human who showed symptoms consistent with HFRS.

MATERIALS AND METHODS

Viral strains and cells. *Hantaan virus* (HTNV) strain 76-118 and SEOV strain SR-11 were used as representative strains of the HTNV and SEOV species, respectively. The THAIV strain Thai749 was a gift from Dr. P.W. Lee of the WHO Collaborating Center for Virus Research for Hantaviruses in Korea. All of the viruses were propagated in Vero cells (clone E6: ATCC C1008) prior to molecular and antigenic characterizations or use in FRNT. The DOBV strains Slovenia[9] and Saaremaa-DOE,[10] SEOV strain Gou3,[11] and HTNV strain *Da Bie Shan virus* (DBSV)-NC167 isolated from *Niviventer confucianus* captured in a mountainous region near Dabishan, Anhui Province, China,[11] were used for antigenic comparisons.

Monoclonal antibodies (MAbs). Clones that produce MAbs directed against the HTNV envelope glycoproteins and N protein were prepared as previously described.[12,13]

Nucleotide sequence determination and phylogenetic analysis. Hantaviruses possess a negative-sense RNA genome that consists of 3 segments, which are designated as large (L), medium (M), and small (S). The L segment encodes the RNA-dependent RNA polymerase. The M segment encodes a glycoprotein precursor that is co-translationally cleaved into the G1 and G2 envelope glycoproteins, and the S segment encodes the nucleocapsid (N) protein.[1] The nucleotide sequence of the M segment has been published, but not in L and S segment. Total RNA was isolated from THAIV-infected Vero E6 cells, and hantavirus-specific cDNA was synthesized, as previously described.[11] To amplify the partial M genome segment that corresponds to nucleotides (nt) 2000–2300, the primer pair THLM1910F, (5′-AAAAGCAGATGTTACAT-3′) and THLM2364R (5′-TTTTCAAGTGACACTT-3′) was used. The entire S genome segment was amplified as 2 overlapping PCR products nt 1–1220 and nt 1025–1885 by using the two primer pairs CS1 (5′-TAGTAGTAGACTCCCT-AAAGAGCTAC-3′) and GS6 (5′-AGCTCIGGATCCAT-ITCAT-3′), as well as GS4 (5′-GAIIGTTGTCCACCAA-CATG-3′) and CS8 (5′-TAGTAGTAGGCTCCCTAAA-AAGACAA-3′).[11,14] The PCR product of the expected size derived from the partial M segment was purified using a PCR purification kit (Qiagen, Hilden, Germany) and sequenced with the same primers that were used for the PCR amplification. The PCR products derived from the S genome segment were cloned into an *E. coli* vector using the Original TA Cloning Kit (Invitrogen, Carlsbad, CA). Two clones of each amplification product were sequenced with M13-forward and -reverse primers. The sequencing reaction was performed with dye terminator reactions using a BigDye Terminator Cycle Sequencing Kit version 3.1 (Perkin Elmer, Applied Biosystems Division, Foster City, CA). The samples were sequenced on model 3100 DNA Sequencing System (Perkin Elmer, Applied Biosystems Division). The sequences obtained from 2 independent clones for each PCR amplification product were found to be identical. Although the almost complete S segment nucleotide sequence (except the extreme 5′- and 3′-termini covered by the amplification primers) was obtained, only the entire N protein coding sequences that allowed unambiguous alignment were used for the phylogenetic analysis.

The sequences were aligned using CLUSTALW[15] with the default parameters. The reliability of the alignment was checked using DotPlot analysis implemented in the BioEdit (Carlsbad, CA) software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The alignment was tested for phylogenetic information by likelihood mapping analysis.[16] In the subsequent phylogenetic analyses, the maximum likelihood (ML) and neighbor-joining (NJ) phylogenetic trees were calculated. To reconstruct the ML phylogenetic trees, a quartet puzzling algorithm implemented in the TREE-PUZZLE 5.2 package[16,17] was applied. The Tamura-Nei and Hasegawa-Kishino-Yano evolutionary models were used for the tree reconstructions. Missing parameters were reconstructed from the datasets. NJ trees with the Tamura-Nei evolutionary model were constructed using the PAUP* 4.0 Beta 10 software package (Sunderland, MA).[18] In addition, bootstrap analysis with 1,000 replicates was performed to evaluate the statistical support of the topology for the derived tree. The resulting evolutionary trees were then visualized using TreeView (Glasgow, UK) v.1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/ treeview.html). The accession numbers of the sequences used in the phylogenetic analysis are listed in the legend to Figure 1. The sequence of the S segment of the THAIV strain Thai749 has been deposited into the GenBank nucleotide sequence database with accession number AB186420.

Indirect immunofluorescent antibody (IFA) assay. Since the HANTADIA assay showed weak agglutination pattern in some of the sera, we also used IFA test for screening test. The indirect immunofluorescent antibody (IFA) assay was performed as described previously.[19] Briefly, acetone-fixed smears of Vero E6 cells infected with hantaviruses were used as antigens. For the antigenic comparison of THAIV with other hantaviruses by using the MAbs (Table 1), HTNV strains 76-118, AMRV-H5, and DABV-NC167, SEOV strains SR-11 and Gou3, DOBV strains Slovenia, and Saaremaa-DOE, and THAIV strain Thai749 were used. Fluorescent isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (H and L chains) (Zymed Laboratories Inc., South San Francisco, CA) was used as the secondary antibody. The serum specimens that showed characteristic fluorescence in the infected Vero cells but negative with uninfected Vero cells were regarded as positive.

Focus reduction neutralization test (FRNT). The endpoint titers of neutralizing antibodies against HTNV strain76-118, SEOV strain SR-11, and THAIV strain Thai749, were determined by FRNT, as described earlier.[20] For this purpose, we selected seropositive sera from human and rodent sera. Human sera and rodent sera derived from trapping point #1 to #13 (Figure 2, Table 2) positive by ELISA were selected. And rodent sera derived from trapping point #14 to #22 positive by both HANTADIA and IFA were selected for further investigation. However a *Rattus rattus* serum from Phetchaburi was not used for FRNT assay because its amount was not suffi-

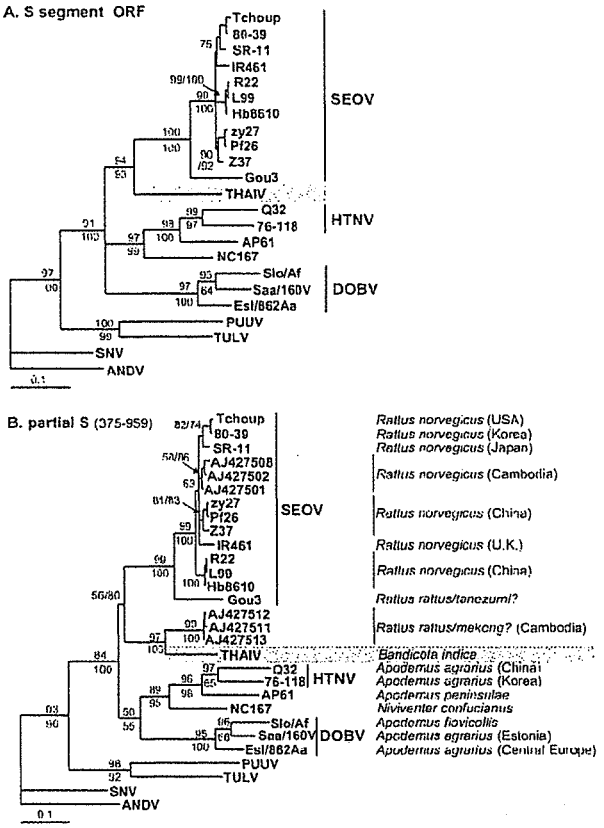

FIGURE 1. Maximum likelihood (ML) phylogenetic trees of THAIV and other Murinae-associated hantaviruses based on: (**A**) complete N protein coding nt sequences (S segment ORF); and (**B**) partial S segment nucleotide sequences of nt 375–959 (585 nts). The ML trees (Tamura-Nei evolutionary model) were calculated using TREE-PUZZLE package. The values above the branches represent PUZZLE support values. The values below the branches are the bootstrap values of the corresponding NJ tree (Tamura-Nei evolutionary model) calculated with the PAUP* software from 1,000 bootstrap replicates. THAIV is marked by a gray box. The S segment sequences that were analyzed included THAIV (AB186420), SEOV/SR11 (M34881), SEOV/Tchoupitoulas (AF329389), SEOV/80-39 (AY273791), SEOV/JR461 (AF329388), SEOV/R22 (AF488707), SEOV/L99 (AF488708), SEOV/Hb8610 (AF288643), SEOV/zy27 (AF406965), SEOV/Pf26 (AY006465), SEOV/Z37 (AF187082), Gou3 (AB027522), HTNV/76-118 (M14626), HTNV/Q32 (AB027097), Amur virus AMRV/AP61 (AB071183), DBSV/167 (AB027523), DOBV/Slo/Af (L41916), DOBV/Esl/862Aa (AJ269550), Saaremaa/160V (AJ009773), PUUV/CG1820 (M32750), Tula virus strain Moravia/5302v (Z69991), Sin Nombre virus strain NM H10 (L25784), and Andes virus strain Chile-9717869 (AF291702). In the lower tree (B), the partial sequences of Cambodian hantavirus strains detected in *Rattus rattus* (AJ427511-AJ427513) and in *R. norvegicus* (AJ427501, AJ427502, AJ427508) were added to the dataset. The natural rodent species (subfamily Murinae) of the corresponding hantavirus strains are listed.

cient. Briefly, 100 μL of serial 2-fold dilutions of serum were mixed with an equal volume of virus suspension containing 200 focus-forming units (FFU) of virus at 37°C for 1 hr. Fifty microliters of the mixture was then inoculated onto Vero E6 cell monolayers in 96-well tissue culture plates (IWAKI 3860-096, Asahi Technoglass Co., Tokyo, Japan). After adsorption for 1 hour at 37°C, the wells were overlaid with medium that contained 1.5% carboxymethyl cellulose. After being incubated for 7 days in a CO_2 incubator, the monolayers were fixed with acetone-methanol (1:1) and dried. The foci of the virus-infected cells were detected by staining with a polyclonal antiserum from a rabbit that was immunized with the truncated N protein (amino acids 1–244) of HTNV, followed by the addition of horseradish peroxidase-labeled goat antibodies and substrate. The FRNT titer was expressed as the reciprocal of the highest serum dilution that resulted in a > 80% reduction in the number of infected cell foci.

Rodent sera and antibody detection. In total, serum samples from 402 different rodents were collected from 22 locations in 19 provinces of Thailand from 1995–1998 (Figure 2, Table 2). Distinction of rodent species examined in the present study followed morphologic criteria including dental morphology and coloration of phage outlined by Corbet and Hill,[21] Musser and Brothers,[22] and Marshall.[7] The blood samples were taken after the animals were anesthetized with CO_2 and taxonomically identified; the weight, sex, and locality of collection were recorded. Then, the animals were euthanized with CO_2. Most of the captured rodents were brought to the Institute. The cadavers were incinerated at the Institute. Serum samples derived from trapping sites #1 to #13 were tested in an indirect IgG ELISA using yeast-expressed His-tagged SEOV, strain 80-39, recombinant N protein.[23] Briefly, polystyrene microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 2 μg/ml recombinant N protein from SEOV diluted in 0.05 M carbonate buffer (pH 9.8). Blocking of the plates was accomplished by the addition of 3% bovine serum albumin (BSA)/0.05% Tween-20 in PBS followed by the addition of rodent serum samples diluted 1/200 with 1% BSA/0.05% Tween-20 and horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (Sigma Chemical Co., St. Louis, MO). To detect immunoreactivity, the *o*-phenylenediamine (OPD) substrate was added, and the reaction was stopped by the addition of 100 μL of 1 M H_2SO_4. Finally, the optical density (OD) was measured at 492 nm (reference, 620 nm). The final OD value for each serum sample was calculated as the difference of the OD values for antigen-containing and antigen-free wells. These final OD values for serum dilutions of 1/200 were regarded as positive if they exceeded the cutoff value of 0.270 determined by investigation of non-infected and experimentally SEOV-infected rats.[23,24] The serum samples derived from trapping sites #14 to #22 were screened using a commercial agglutination test based on inactivated HTNV antigen (HANTADIA®; Korea Green Cross Corp., Seoul, Korea) and an indirect immunofluorescent antibody (IFA) test. In HANTADIA screening, sera were screened by the manufacturer's instructions at 1:40 dilution. Serum specimen that showed clear agglutination was regarded as positive. In IFA test, the sera were examined at 1:40 dilution with HTNV strain 76-118-infected Vero E6 cell smears as antigen. As negative control, each serum sample was tested with uninfected Vero E6 cells. The serum specimen that showed characteristic fluorescence in the infected Vero E6 cells but negative with uninfected Vero E6 cells was regarded as positive. Antibody-positive sera from both screenings were confirmed by Western blotting using recombinant hantavirus N proteins of HTNV strain 76118 as previously described.[23,25,26] As positive controls, 3 serum samples from Wistar rats that were experimentally in-

TABLE 1
Antigenic profiling with N-, G1-, and G2-specific MAbs of THAIV and other murinae-associated hantaviruses*

Proteins	Epitope	MAbs	HTNV			SEOV		DOBV		THAIV
			HTNV 76118	AMRV H5	DBSV NC167	SEOV SR-11	SEOV Gou3	DOBV Slovenia	DOBV Saaremaa	THAIV Thai749
N	Cross-reactive	ECO2	+++	+++	+++	+++	+++	+++	+++	+++
	Genus-common	E5G6	+++	+++	+++	+++	+++	++	++	+++
	HTNV-specific	BDO1	+++	+++	+++	-	-	-	-	-
	SEOV-specific	DCO3	-	-	-	+++	+++	-	-	-
G1/G2	G1a	6D4	+++	+++	+++	-	-	+++	+++	+++
	G1b	3D5	+++	+++	+++	-	-	-	-	-
	G2a	HCO2	+++	+++	+++	+++	+++	-	-	+++
	G2b	EBO6	+++	+++	+++	+++	+++	±	±	-
	G2c	11E10	+++	+++	+++	-	-	+++	-	-
	G2d	3D7	+++	++	+++	+++	+++	+++	+++	+++
	G2e	20D3	+++	+++	-	+	+	++	++	+++
	G2f1	1G8	+++	++	+++	+++	+++	+++	+++	+++
	G2f2	7G6	+++	++	+++	+++	+++	++	+++	+++

Binding profiles of clones data not shown in this table were basically same results as representative clones and previous reports.[1] All the used clones were listed as below. Cross-reactive clones for N protein: ECO2, FDO3, KAO6, ECO1, GBO4, C16D11, and F23A1; Genus-common epitope binding clone: E5G6; HTNV-specific clones for N protein: BDO1, C24B4, and G5; SEOV-specific clone for N protein: DCO3.

Clones for glycoprotein epitope G1a: 6D4, 8B6, and 10F11; G1b: 3D5, 2D5 and 16D2; G2a: HCO2 and 16E6, G2b: EBO6; G2c: 11E10; G2d: 3D7; G2e: 20D3, 17G6 and 5B7; G2f1: 1G8, 8E10, 1C6, 23G10-2, and 3B6; G2f2: 7G6, 23G10-1, and 18F5. Designations: -, $< 10^2$; +, 10^2; ++, 10^3; +++, 10^4; ±, weak positive reaction at dilution of 1:100.

fected with SEOV strain SR-11 were used.[26] As negative controls, sera from 5 wild-trapped, non-infected rats from Japan were used.[24]

Human sera and methods for antibody detection. Screening for anti-hantavirus IgG and serotyping were performed by ELISA tests, as previously described,[20] using recombinant entire and truncated N protein antigens expressed by recombinant baculovirus. Briefly, serum specimens were screened with the dilution of 1:200. As a negative control antigen, bornavirus P24 antigen expressed by baculovirus was used. Recombinant N proteins of HTNV (strain 76-118), and PUUV (strain Sotkamo) and truncated N proteins of HTNV (strain 76-118), SEOV (strain SR-11), and DOBV (strain Saaremaa-DOE) were expressed from baculovirus vectors. The screening for virus-reactive IgM was performed with the μ-capture ELISA, as described previously.[27] Positive results were confirmed by IFA testing using SEOV-infected Vero E6 cell antigen and by Western blotting using recombinant HTNV antigen. Three types of positive control sera from HFRS patients who had been previously diagnosed by FRNT as being infected with HTNV, SEOV, and PUUV, and negative human control sera (NHS), which were confirmed to contain no antibodies against hantaviruses, were used.[20,28]

RESULTS

Genetic characterization of Thailand virus. The nucleotide sequences of the entire M genome segment and partial S genome segment of the THAIV strain Thai749 have been published (GenBank accession numbers L08756 and U00471[5]). Partial M segment sequence of the THAIV obtained in this study was completely identical with the published sequence. To characterize genetically the THAIV strain Thai749 in more detail, we cloned and sequenced entire S genome segment except primer binding region (GenBank accession number AB186420). The sequences of 2 independent clones for each of the PCR amplification products were found to be identical. The deduced amino acid sequence identity on comparison of the N protein of THAIV to those of SEOV, HTNV, and DOBV are calculated as 86.5%, 83.7%, and 81.6%, respectively. The previously determined values for sequences of THAIV glycoprotein precursors[5] showed amino acid sequence identity to those of SEOV, HTNV, and DOBV as 73.3%, 71.3%, and 71.2%, respectively. Thus, the N protein amino acid sequence information also meet one of the criteria set forth in the Eighth Report of the International Committee on Taxonomy of Viruses for species demarcation within the genus *Hantavirus* (more than 7% difference).[1,29] The phylogenetic analysis (Figure 1A) based on the nucleotide sequence of the N protein-encoding open reading frame (ORF) of the S genome segment revealed that THAIV was clearly placed in a distinct lineage within a single cluster with SEOV, HTNV, and DOBV, which are associated with the rodent reservoirs classified into the murid subfamily *Murinae*. Since *B. indica* is classified to the *Murinae* subfamily, the observed lineage of THAIV is in accordance with the host-virus co-evolution theory for hantaviruses.[30,31] As shown in Figure 1B, phylogenetic analysis based on a partial nucleotide sequence (nt 375–959) in the central region of the S segment, which contains the highly variable region, reveals that THAIV is most closely related to Cambodian virus strains isolated from *R. rattus*.[32]

Antigenic characterization of Thailand virus using monoclonal antibodies. To clarify the antigenic characteristics of THAIV, 34 MAbs, including 12 against the N protein and 22 against the G1 or G2 envelope proteins, were used to compare the antigenic profiles of the THAIV prototype strain Thai 749 and other hantaviruses using IFA (Table 1). The antigenic profiles of HTNV strains 76118 and Amur virus (AMRV)-H5 were taken from a previous report.[33] Among the MAbs directed against the N protein, cross-reactive clones to HTNV, SEOV, and DOBV-types were also reactive against THAIV. On the other hand, the HTNV-type specific and SEOV-type specific clones for N protein were not reactive against THAIV. Similarly, HTNV-type specific anti-G1 MAb (3D5) did not react to THAIV. However, the rest of clones showed variable cross reactivities among the 4 types of viruses. Therefore, in spite of the close antigenic relationships between hantaviruses that are associated with *Murinae* reservoir hosts, the antigenicity of THAIV was distinct from the

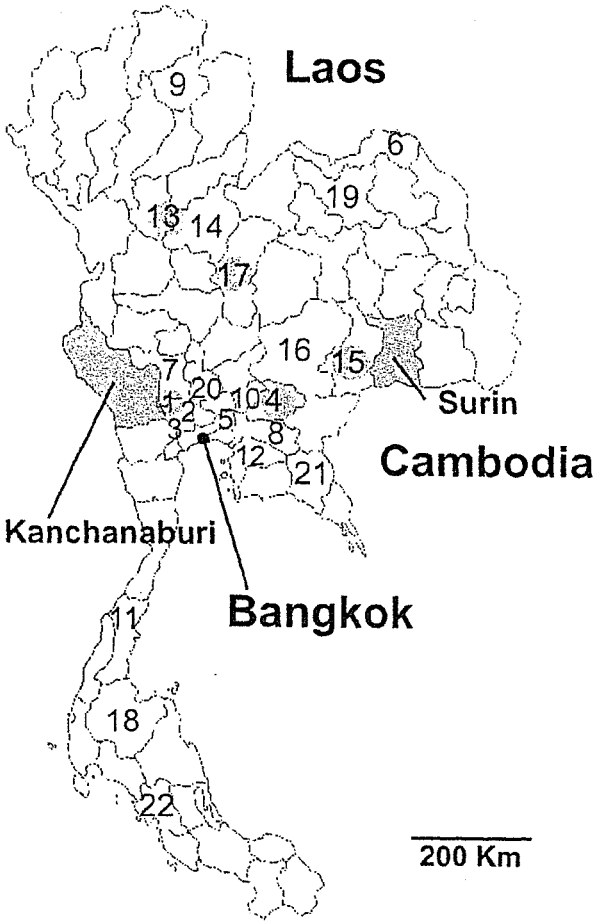

FIGURE 2. Map of Thailand showing the localization of rodent trapping sites. The numbers of the trapping sites correspond to those given in Table 1. The locations of the trapping sites where anti-hantavirus-positive rodents were captured are shown as gray circles. The geographical origin of patient #277 in Surin Province is shown as a gray area. The locality of collection of the *Bandicota indica* used for isolation of THAIV strain Thai749, in the western province Kanchanaburi is given as gray area.

other Murinae-associated hantaviruses. These results were corresponding to the previous report from Dr. Chu et al. [3]

Serological survey of rodent sera. In total, serum samples from 402 rodents captured at 22 different sites (Figure 2) were examined for IgG reactive against hantavirus antigens. Serological screening was carried out by ELISA or with an agglutination kit (HANTADIA). As shown in Table 2, 7 of 402 (1.7%) serum samples were antibody-positive. Of the 7 seropositive specimens, 5 were derived from *B. indica*, one from *B. savilei*, and one from *Rattus rattus*. The 5 provinces in which the seropositive rodents were located (i.e., Nakhon Pathom, Prachin Buri, Phitsanulok, Buri Ram, and Phetchabun) are distributed close to Bangkok in the eastern and northern parts of Thailand (Table 2, Figure 2).

To characterize the apparent homologous virus, 5 positive *Bandicota* sera were selected, and FRNT investigations were performed (Table 3). All of the rodent sera showed the highest FRNT titers to THAIV, which indicates that THAIV or THAI-like viruses exist among rodents in Thailand. Two

TABLE 2
Trapping sites, collected rodent species and seropositivity for hantavirus

Site no.	Trapping site	Rodent species	No. of seropositives/ No. of animals tested
1	Nakhon Pathom/Bang Len	*Rattus rattus*	0/9
		Bandicota indica	**1/83**
2	Nakhon Pathom/Nakhon ChaiSi	*R. rattus*	0/1
3	Nakhon Pathom/Sam Phran	*R. rattus*	0/1
4	Prachin Buri	*R. losea*	0/7
		B. indica	**2/18**
5	Bangkok Metropolis	*R. norvegicus*	0/16
		R. rattus	0/9
6	Nong Khai	*R. rattus*	0/1
7	Suphan Buri	*R. argentiventer*	0/11
8	Chanchoengsao/Bang Nam Prieo	*R. norvegicus*	0/34
		R. rattus	0/6
		B. indica	0/34
9	Phayao	*R. rattus*	0/1
10	Nakhon Nayok	*R. rattus*	0/1
11	Chumphon/Bang Son (Pathiu)	*R. tiomanicus*	0/97
12	Chon Buri	*B. indica*	0/2
		B. savilei	0/1
		R. rattus	0/2
		R. exulans	0/1
13	Phitsanulok/Phrom Piram	*R. argentiventer*	0/19
		R. losea	0/4
		B. savilei	**1/3**
14	Phitsanulok	*B. indica*	0/7
		R. rattus	0/6
15	Buri Ram	***B. indica***	**2/3**
		R. rattus	0/2
16	Nakhon Ratchasima	*B. indica*	0/2
		R. rattus	0/1
17	Phetchabun	*B. indica*	0/3
		R. rattus	**1/1**
18	Surat Thani	*R. rattus*	0/2
19	Udon Thani	*R. rattus*	0/1
		R. exulans	0/2
20	Ayutthaya	*B. indica*	0/1
		R. rattus	0/1
		R. exulans	0/2
21	Chanthaburi	*R. rattus*	0/1
		R. norvegicus	0/2
22	Trang	*R. rattus*	0/2
		R. exulans	0/2
	All	***B. indica***	5/152 (3.3%)
		B. savilei	1/5 (20.0%)
		R. rattus	1/48 (2.1%)
		R. exulans	0/7
		R. norvegicus	0/52
		R. losea	0/11
		R. argentiventer	0/30
		R. tiomanicus	0/97
	Total		7/402 (1.7%)

The groups in which the positive rodents were detected are shown by the bold-faced type.

other positive sera, one from *B. indica* and one from *R. rattus*, were not available for the FRNT due to an insufficient amount of serum.

Serological survey of human sera. A total of 260 paired sera were obtained from 260 patients who were clinically diagnosed with leptospirosis but were serologically negative for *Leptospira* antigens. Two paired sera (#53 and #54, #277 and #277/2004) showed positive reactions against the HTNV antigen but negative or very low reactivity against the PUUV antigen (Figure 3A). The ELISA OD values of anti-hantavirus IgG in serum #53 and #54 were 0.309 and 0.398,

TABLE 3

Analysis of human and rodent serum samples in focus reduction neutralization test (FRNT) using *Hantaan* virus (HTNV), Seoul virus (SEOV), and *Thailand* virus (THAIV)

Serum specimen/antiserum	Reciprocal end-point titer† against		
	HTNV	SEOV	THAIV
A172 *(Bandicota indica)*	< 40	< 40	**80**
Bi65 *(B. indica)*	< 40	< 40	**≥ 1280**
Bi74 *(B. indica)*	< 40	< 40	**≥ 1280**
Bi324 *(B. indica)*	< 40	< 40	**80**
Bs355 *(B. savilei)*	< 40	< 40	**160**
Anti-HTNV/mice	**≥ 1280**	< 40	< 40
Anti-SEOV/rat	< 40	**≥ 1280**	80
Negative sample of *B. indica*	< 40	< 40	< 40
#277	40	< 40	**160**
Anti-HTNV	**640**	< 40	< 40
Anti-SEOV	80	**640**	160
NHS‡	< 40	< 40	< 40

† The highest neutralizing antibody titer for each serum is given in bold.
‡ Serum sample from a healthy human individual.

respectively. The virus-specific IgM was not detected (Figure 3A, 3B). Therefore, this patient may have been infected with a hantavirus many years ago and was suffering from an illness that was unrelated to recent hantavirus infection. Serum #277 contained high concentrations of HTNV-reactive IgM and IgG (Figure 3A, 3B). The #277/2004 serum, which was collected 12 months after the onset of disease, showed high IgG concentration but quite lower IgM concentration. The presence of anti-hantavirus antibodies in serum #277 and #277/2004 was also confirmed by IFA testing using SEOV-infected Vero E6 cells and by Western blotting using recombinant HTNV N protein antigen (data not shown). The detection of HTNV-reactive IgM in patient serum #277 in acute phase but not in convalescent phase may represent an indication of an hantavirus infection.

The serotyping of serum #277 by ELISA revealed reactivities to the truncated N proteins of HTNV, SEOV, and DOBV. However, unlike the positive control sera, serum #277 was equally reactive against the 3 test antigens (Figure 3C). This may indicate that the patient with serum #277 was probably infected with a hantavirus other than HTNV, SEOV, and DOBV. To further characterize the antibody response of serum #277, neutralizing capacity against HTNV, SEOV, and THAIV was tested using FRNT (Table 3). The results indicated that the patient with serum #277 was infected with either THAIV or a THAI-like virus, since the neutralizing antibody titer against THAIV was at least 4-fold higher than that against HTNV or SEOV.

The clinical profile of the patient with serum #277 was consistent with HFRS. The male patient was a 26-year-old farmer from Surin province in northeastern Thailand who was admitted to a mobile "fever unit" with a 40°C fever that had developed over the previous days. The physical examination on admission showed a well orientated patient who suffered from headache, abdominal pain, and conjunctival suffusion. Urine analysis displayed a proteinuria, glucosuria, erythrocyturia, and leukocyturia. The serum level of the alanine aminotransferase was 110 IU/l, the aspartate aminotransferase level was 240 IU/l, and the alkaline phosphatase level was 480 IU/l. The patient showed neither hemorrhages nor oliguria. The serological tests performed for leptospirosis, dengue fever, influenza, and scrub typhus were negative.

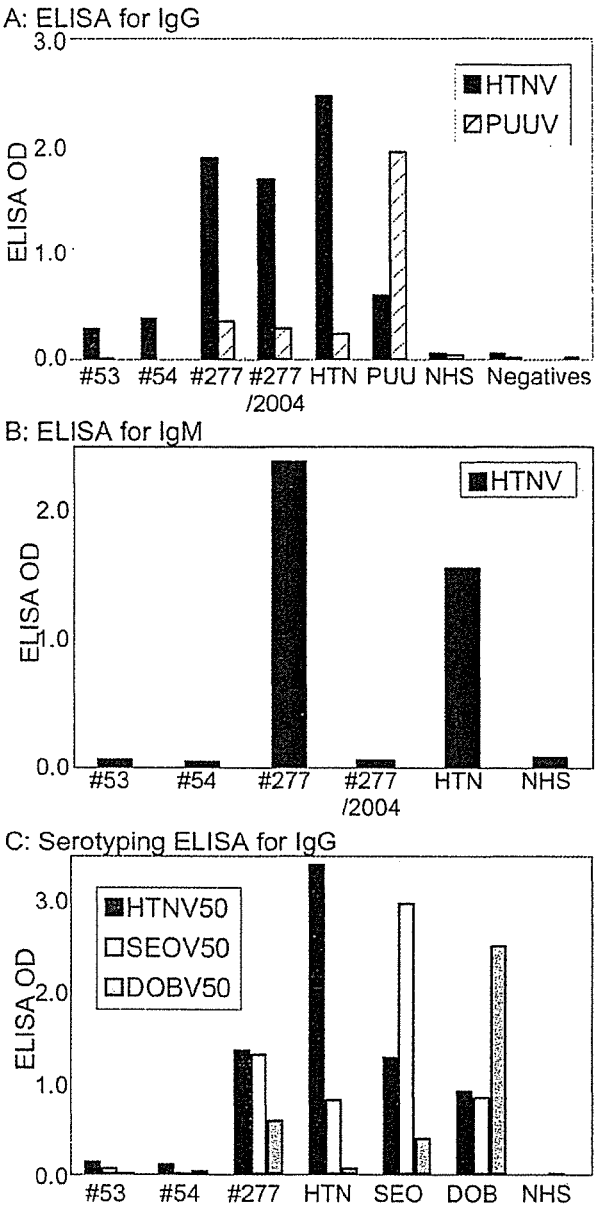

FIGURE 3. Serological screening of patient sera obtained in Thailand. (**A**) Detection of anti-hantavirus IgG in patient sera #53, #54, #277, and #277/2004 by ELISA using the recombinant N protein antigens of HTNV (closed bar) and PUUV (hatched bar). HTN and PUU are positive control sera from patients who were previously confirmed to be infected with HTNV in China and with PUUV in Sweden, respectively. NHS represents a human serum sample that was confirmed as negative for hantavirus-specific antibodies. Typical negative reactions in the initial screening assay are shown as negative. (**B**) Detection of anti-hantavirus IgM by μ-capture ELISA using the recombinant N protein antigen of HTNV. HTN is a positive control serum sample of the acute phase from a patient previously confirmed as being infected with HTNV. (**C**) Serotyping ELISA for human sera #53, #54, and #277 using truncated N antigens (amino acids 50-429) of HTNV (HTNV50), SEOV (SEOV50), and DOBV (DOBV50). Human sera HTN, SEO, and DOB were used as positive controls in the assay and have been characterized previously.

All the methods used showed basically the same tree topology. Therefore, only the ML trees with the Tamura-Nei evolutionary model are shown. We obtained a total of 260 paired sera (acute phase and convalescent phase) from different patients who had a fever of unknown etiology and were found to be seronegative for leptospirosis, dengue fever, influenza, and scrub typhus. The sera were collected in Surin Province of Thailand (Figure 2) in 2002 (454 sera), 2003 (65 sera), and 2004 (1 serum).

DISCUSSION

To further characterize the genetic and antigenic relatedness of THAIV to other Asian hantavirus species, first we cloned and sequenced the almost entire S genome segment of THAIV strain Thai749. Our sequence and phylogenetic analysis based on the nucleotide sequence of the N-protein-encoding ORF on the S segment revealed the same conclusions as previously drawn from complete M segment analyses: THAIV is most closely related to the SEOV species but different enough to appear as a distinct branch on the phylogenetic tree.[5,11] The different aa sequence similarities are reflected also in the reactivity of N-, G1-, and G2-specific MAbs with the corresponding proteins of THAIV and other hantaviruses. In general, our IFA reactivities of all anti-G1 and anti-G2 MAbs with Thai749 are in line with data of ELISA investigations published previously. In contrast, the reactivity of these MAbs in hemagglutination inhibition assay and especially plaque reduction neutralization test (PRNT) differed markedly to our IFA data, most likely due to the differences of the test formats.[3] The definition of THAIV as a distinct species was based on its association to a unique rodent species (i.e., *B. indica*).[4] Recently, THAIV genome was amplified by RT-PCR from *B. indica* captured in central Thailand (personal communication from Alexander Plyushin). In addition, the 2-way cross-neutralization test with sera from a patient and naturally infected bandicoot rats showed more than a 4-fold difference. This is in line with data of PRNT investigations of a rat anti-Thai749 immune serum with a large panel of strains of different hantavirus species.[3] Therefore, this report provides additional support for defining THAIV as a distinct species among the hantaviruses.

Schmaljohn et al.[34] reported that the N proteins of HTNV, SEOV, and PUUV have an overall amino acid sequence identity of 50%. However, certain regions of the N protein, such as that spanning amino acid residues 240–310 display only a low level of sequence identity (about 11%) to each other. Therefore, the corresponding N protein-encoding sequence between nt 760–970 is considered as variable region among hantaviruses. By phylogenetic analysis based on nucleotide sequences between positions 375–959 of S genome segment, which contains the variable region, we found a close genetic association of THAIV with the *R. rattus*-associated Cambodian virus strains. Therefore, it is suggested that THAIV and closely related viruses occur throughout Indochina.

The present study extends our knowledge of the geographical distribution and natural host relationships of hantaviruses indigenous to Thailand. A serological survey of rodent samples originating from 22 provinces of Thailand resulted in the identification of hantavirus-reactive samples of *B. indica* from 3 different provinces located in the central plains and northeastern parts (Khorat plateau) of the country. Determination of the endpoint titers of these sera in neutralization assays using HTNV, SEOV, and THAIV revealed infections with THAIV or a THAI-like virus. Similarly, a serum sample originating from *B. savilei* confirmed the occurrence of THAIV or a THAI-like virus in an additional province in the north of Thailand. However, as no viral genetic material is available from *B. savilei* we can not exclude that the detection of THAIV-reactive antibodies is the result of a spill over infection that might have occurred in this region due to a high infectious pressure of this virus. Our findings on the geographical distribution of THAIV overlap with the observations of Nitatpattana et al.,[35,36] who found hantavirus-infected giant bandicoot rats in the central plains as well as in 3 northeastern provinces of Thailand (Khon Kaen, Buri Ram, Surin). A majority of the hantavirus-positive rodents were collected from rice field habitats.[36] In the latter study the highest seroprevalence was observed in giant bandicoot rats from Khon Kaen, an area that lies at the center of the Khorat plateau, whereas comparative quantities of animals collected further east, from Nakhon Phanom and Kalasin, were all hantavirus negative. Unfortunately, we were not able to collect serum samples of bandicoot rats from southern Thailand. Interestingly, a recent serological study conducted in neighboring Cambodia employing HTNV as antigen (660 rodents) found roof rats, Norway rats, and unidentified *Rattus* species infected with hantaviruses closely related to SEOV, but none of 75 bandicoot rats and 183 Polynesian rats (*Rattus exulans*).[32] Therefore, search for THAIV or THAI-like viruses should be extended to southern provinces as well as neighboring Cambodia.[32] THAIV is antigenically cross reactive to HTNV and SEOV. Therefore, previous seroepidemiological studies with the heterologous viruses would detect the prevalence of THAIV infection with the same sensitivity as with THAIV antigen. For further epidemiologic studies, serological typing would certainly profit to elucidate the situation of THAIV infection. Virus isolation and genome amplification from *B. indica* originating from different provinces in Thailand have not been attempted so far, but would be very important to extend our knowledge on the distribution and variability of THAIV and THAI-like viruses in Indochina.

Serological detection of THAIV-reactive antibodies in patients with fever of unknown origin from Surin province confirmed the circulation of THAIV or THAI-like viruses in Thailand. Particularly, results of patient #277 suggest that THAIV or THAIV-like virus causes HFRS. We interpret the close geographical proximity of this THAIV-reactive human sample to the seropositive samples from bandicoot rats as a first indication of a potential epidemiologic relationship. Elwell et al.[4] reported that people living in an area where seropositive giant bandicoot rats were trapped showed a higher seroprevalence than those living in a low prevalence area. Nitatpattana et al.[36] observed that *B. indica* was the species with the highest prevalence of anti-hantavirus antibodies in a study on rodents from northeastern Thailand. Similarly, our study revealed the greater bandicoot rat as the species with the highest seroprevalence against hantavirus in general, and THAIV in particular (3.3% in *B. indica*), and identified the lesser bandicoot rat (*B. savilei*) as a potential new host for THAIV. In combination, this suggests that a higher prevalence of infection of bandicoot rats as such already poses a higher risk for humans to become infected with THAIV than with other hantaviruses from other rodent species. This especially applies to rural areas, where both commensal (e.g., ro-

dent species inhabiting houses) as well as field rodents like bandicoot rats live in close association with humans.[37] In the case of THAIV this is possibly aggravated by the fact that 50–80% of residents in some rural areas trap, cook, and eat *B. indica*.[1,37]

Although a recent publication attributed a first clinical case to hantavirus infection in Thailand,[8] the causative hantavirus species was not further characterized. In our study we identified a patient who developed a clinical profile similar to that of HFRS with high concentrations of IgM and IgG to HTNV by an initial screening of various human sera. Because this serum showed lower titers to HTNV, SEOV, and DOBV antigens compared with virus-specific human positive control sera, and, importantly, contained significant concentrations of virus-neutralizing antibodies against THAIV, these observations suggest that THAIV or a THAI-like virus caused this infection. Furthermore, our FRNT results show close similarities between the particular patient serum and sera from rodents of the genus *Bandicota*, especially *B. indica* representing a host of THAIV.[4] In most hantaviral disease cases, both IgM and IgG to hantavirus are positive at the onset of clinical disease.[38] The reduction of the titer of HTNV-reactive IgM in a follow-up serum sample from convalescent phase of the patient may indicate that THAIV or a related virus is a causative agent of HFRS. However, since hantavirus-reactive IgM might be detected up to 6 months after onset of disease,[39] the possibility that the febrile illness might be caused by infection with other pathogen could not be excluded. Therefore, further epidemiologic study is needed to find out similar patients with hantavirus antibody. Nevertheless, the results of Supputthamongkol et al.[8] and our study indicate that human disease caused by hantaviruses may be more prevalent in Thailand than anticipated earlier, because clinical cases may have been confused with leptospirosis, a rodent-transmitted disease that causes similar symptoms in humans like conjunctival suffusion, hemorrhagic manifestation, renal failure, and hepatic dysfunction.[40] Further epidemiologic studies, including virus isolation, are needed to elucidate the relationship between fevers of unknown origin, presence of THAIV or THAI-like viruses in rodents, and potential transmission from rodents to humans.

In conclusion, we have demonstrated that distribution of *Bandicota*-associated THAIV or THAI-like viruses extends from the central plains of Thailand to the north and northeast. Our genetic and serological studies confirmed the definition of THAIV as a distinct hantavirus species. Moreover, our data suggest that THAIV, besides HTNV and SEOV, may represent an additional causative agent of HFRS in Asia. Recently, we found anti-hantavirus antibody-positive sera both in humans and rodents in Vietnam.[41] Molecular, epidemiologic, and serological studies on hantaviruses in rodents and humans have also been reported from Cambodia and Indonesia.[32,42] Taken together, this indicates a wide distribution and potentially high diversity of hantaviruses in Southeast Asia calling for further studies on human hantavirus infections, its rodent reservoirs, and possible transmission routes.

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Soochong Virus: An Antigenically and Genetically Distinct Hantavirus Isolated From *Apodemus peninsulae* in Korea

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Hantaan (HTN) virus, the etiologic agent of clinically severe hemorrhagic fever with renal syndrome (HFRS), was first isolated in 1976 from lung tissue of a striped-field mouse (*Apodemus agrarius*) captured in Songnae-ri, Gyeonggi Province, Republic of Korea. Found primarily in mountainous areas, the Korean field mouse (*A. peninsulae*) is the second-most dominant field rodent species found throughout Korea. A new hantavirus, designated Soochong (SOO), was isolated in Vero E6 cells from four *A. peninsulae* captured in August 1997 at Mt. Gyebang in Hongcheon-gun, Mt. Gachil, Inje-gun, Gangwon Province, and in September 1998 at Mt. Deogyu, Muju-gun, Jeollabuk Province. The entire S, M, and L genomic segments of SOO virus, amplified by RT-PCR from lung tissues of seropositive *A. peninsulae* and from virus-infected Vero E6 cells, diverged from HTN virus (strain 76–118) by 15.6%, 22.8%, and 21.7% at the nucleotide level and 3.5%, 9.5%, and 4.6% at the amino acid level, respectively. Phylogenetic analyses of the nucleotide and deduced amino acid sequences, using the maximum parsimony and neighbor-joining methods, indicated that SOO virus was distinct from *A. agrarius*-borne HTN virus. SOO virus shared a common ancestry with Amur virus from Far East Russia, as well as with H5 and B78 hantaviruses, previously isolated from HFRS patients in China. Cross-focus-reduction neutralizating antibody tests showed that SOO virus, which is the first hantavirus isolated in cell culture from *A. peninsulae*, could be classified as a new hantavirus serotype. ***J. Med. Virol. 78:290–297, 2006.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: *Apodemus peninsulae; hantavirus;* HFRS; soochong virus

INTRODUCTION

Hantaviruses, members of the family *Bunyaviridae*, are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) [Lee et al., 1978; Nichol et al., 1993]. They are negative-sense, single-stranded RNA viruses possessing large (L), medium (M), and small (S) genomic segments that encode the viral polymerase, envelope glycoproteins (G1, G2), and nucleocapsid (N) protein, respectively [Schmaljohn et al., 1986, 1987; Schmaljohn, 1990]. Hantaan (HTN) virus, the etiologic agent of clinically severe HFRS in Far East Asia and Russia, was first isolated from lung tissues of the striped-field mouse (*Apodemus agrarius*) captured in Songnae-ri, Gyeonggi Province, Korea [Lee et al., 1978].

Hantaviruses show co-evolution and co-speciation with specific rodent species, for example, HTN virus with *A. agrarius*, Seoul (SEO) virus with *Rattus norvegicus* and *R. rattus*, Puumala (PUU) virus with *Clethrionomys glareolus*, Prospect Hill (PH) virus with *Microtus pennsylvanicus*, Dobrava–Belgrade (DOB)

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virus with *A. flavicollis*, Tula (TUL) virus with *M. arvalis*, Khabarovsk (KBR) virus with *M. fortis*, Sin Nombre (SN) virus with *Peromyscus maniculatus*, New York (NY) virus with *P. leucopus*, Black Creek Canal (BCC) virus with *Sigmodon hispidus*, Bayou (BAY) virus with *Oryzomys palustris*, El Moro Canyon (ELMC) virus with *Reithrodontomys megalotis*, and Andes (AND) virus with *Oligoryzomys longicaudatis* [Lee et al., 1978, 1982, 1998; Brummer-Korvenkontio et al., 1980; Avsic-Zupanc et al., 1992; Gligic et al., 1992; Nichol et al., 1993; Plyusnin et al., 1994; Song et al., 1994, 2004].

HTN virus is the primary etiologic agent of HRFS in Korea. Approximately 100–300 HFRS cases are reported annually with a mean mortality rate of 4.5%. About 70% of these HFRS patients are infected with HTN virus, 20% with SEO virus, and the remaining 10% of cases by unidentified agents. To identify other HFRS-causing hantaviruses, surveillance of populations of Korean field mice (*A. peninsulae*) was performed. The Korean field mouse, which inhabits mountainous areas throughout Korea, is the second-most dominant field-rodent species in Korea. We now report the isolation and characterization of Soochong (SOO) virus, an antigenically and genetically distinct hantavirus isolated from Korean field mice captured in Korea.

MATERIALS AND METHODS

Rodent Trapping and Serology

Korean field mice were live caught at Mt. Gyebang (1,577 m) in Hongcheon-gun and Mt. Gachil (1,241 m) in Inje-gun, Gangwon Province, on August 6–7, 1997, and Mt. Deogyu (1,614 m) in Muju-gun, Jeollabuk Province, on September 16–19, 1998 (Fig. 1). Rodent sera were screened for IgG antibodies against hantavirus by the indirect immunofluorescent antibody (IFA) technique, using slides spotted with HTN virus-infected Vero E6 cells. Lung and spleen tissues were frozen at −70°C until used for virus isolation and RNA extraction.

Virus Isolation

Subconfluent monolayers of Vero E6 cells (ATCC CRL 1586), grown in 25-cm^2 flasks, were inoculated with 5% suspensions of lung and spleen tissues prepared in DMEM without fetal bovine serum (FBS). Inocula were allowed to adsorb for 2 hr followed by centrifugation for 2 hr at 670*g* at 25°C. Subsequently, the cells were maintained with DMEM supplemented with 5% heat-inactivated FBS. Cells were subcultured at 10- to 14-day intervals, at which time an aliquot of cells was examined for hantavirus antigens by IFA, using convalescent-phase sera from patients with Korean hemorrhagic fever, and rat and mouse sera specific for HTN, SEO, PUU, and PH viruses. Hantavirus antigen-positive Vero E6 cell cultures were examined for hantaviral genomic sequences by RT-PCR using a consensus primer sets for HTN-SEO viruses [Xiao et al., 1991, 1992].

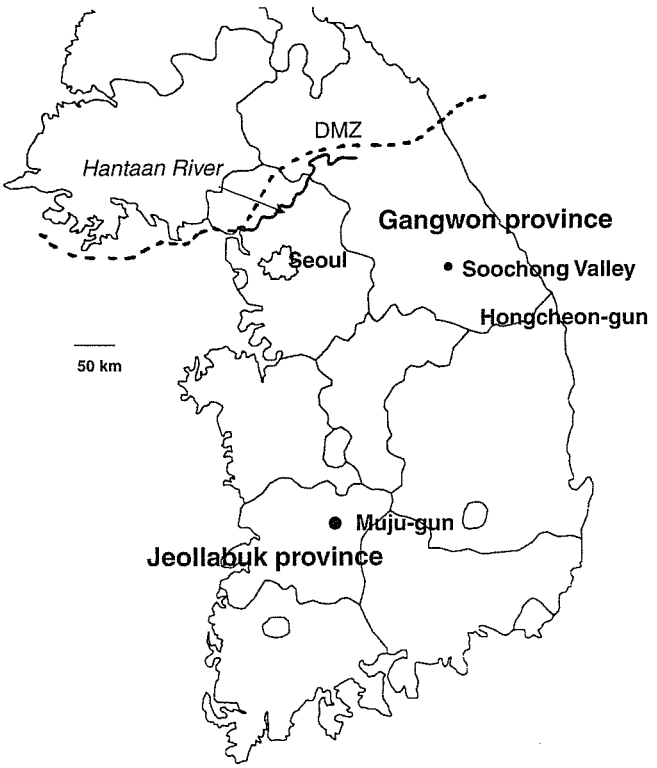

Fig. 1. Map of Korea, showing *A. peninsulae* capture sites, which led to the isolation of Soochong virus.

Antigenic Characterization of Hantaviruses by IFA Using Monoclonal Antibodies (MAbs)

A panel of MAbs that recognize the glycoproteins G1 and G2 and nucleocapsid protein of HTN or SEO viruses was employed in a standard IFA format [Arikawa et al., 1989; Yoshimatsu et al., 1996]. Briefly, Vero E6 cells, infected with hantaviruses (HTN 76–118, H5, and SOO-3) and cultured for 5 days, were trypsinized, suspended in MEM with 5% FBS and spotted onto 24-well slides. After incubation at 37°C for 4 hr, the slides were fixed with cold acetone, air-dried and used as antigen slides. Serially diluted MAbs (1:1 to 1:1,000 for MAbs that were derived from culture supernatant and 1:100 to 1:100,000 for MAbs that were derived from mouse-derived ascitic fluid) were spotted onto virus-infected Vero E6 cells and incubated for 1 hr at 37°C. After three washes with phosphate-buffered saline (PBS), fluorescein isothocyanate (FITC)-conjugated goat antibody to mouse IgG (ICN Pharmaceuticals, Inc., Aurora, OH) was added, incubated at 37°C for 1 hr, followed by three washes with PBS. Specific binding was detected by fluorescence microscopy.

Focus-Reduction Neutralization Test

Endpoint titers of neutralizing antibodies were determined by the focus-reduction neutralization test (FRNT). Mouse immune sera, which were prepared against H5, Bao14, and HTN 76–118 strains, and sera from antibody-positive *A. peninsulae* were used to

analyze the serological relationships between SOO and other hantaviruses [Lokugamage et al., 2002, 2004]. H5 and B78, isolated from Chinese HFRS patients, and Bao14, isolated from *A. agrarius* in China, were previously known as HTN virus strains [Liang et al., 1994; Wang et al., 2000; Lokugamage et al., 2002]. Serially two-fold dilutions of sera (100 μl) were incubated for 1 hr at 37°C with equal volumes of stock viruses (100 focus-forming U/100 μl); 100 μl of the virus-serum mixtures were then inoculated onto Vero E6 cell monolayers grown in eight-well slides. After adsorption for 1 hr at 37°C, the inocula were removed and MEM containing 1.5% carboxymethyl cellulose (CMC) was layered onto the cells. The slides were incubated in a CO_2 incubator at 37°C for 5–7 days. Monolayers were then washed with PBS, fixed with methanol at room temperature, and air-dried. Mouse immune serum to HTN 76–118 strain was added to the fixed Vero E6 cells, incubated for 1 hr at 37°C, followed by three washes with PBS. FITC-conjugated antibody to mouse IgG was added to the cells and incubated at 37°C for 1 hr. FITC-stained foci were counted under a fluorescence microscope. The FRNT titer was determined as the highest dilution of serum that showed 80% or greater reduction of focus formation.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA, extracted using RNAzol (GIBCOBRL, Gaithersburg, MD) from lung tissues of 16 hantavirus-seropositive *A. peninsulae* captured in 1997 and 1998 and from Vero cells infected with four strains of SOO virus, was reverse transcribed using the superscript II RNase H^- reverse transcriptase kit (GIBCOBRL). SOO virus sequences were then amplified by RT-PCR, using previously described and newly designed oligonucleotide primers [Xiao et al., 1991, 1992; Song et al., 2000, 2004]. These primers afforded the amplification of the entire S, M, and L segments of SOO virus. PCR products were cloned using the TOPO-TA cloning system (Invitrogen Corp., San Diego, CA), while plasmid DNA was purified by the QIAprep-spin Plasmid kit (QIAGEN, Inc., Chatsworth, CA). DNA sequencing was performed in both directions from at least three clones of each PCR product, using the dye primer cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) on an automated sequencer (Model 373A).

PCR Amplication of Mitochondrial DNA (mtDNA)

Total DNA was extracted from rodent liver tissues using the QIAamp Tissue Kit (QIAGEN). To study the phylogenetic relationship of *A. peninsulae* from various geographic regions, the cytochrome b region of mtDNA was amplified by PCR, using previously described universal primers that permitted amplification of 482-bp products [Bibb et al., 1981; Smith and Patton, 1991]. PCR was carried out in 50-μl reaction mixtures, containing 200 μM dNTP and 1.25 U of rTaq polymerase (Takara, Shiga, Japan). PCR fragments were amplified with an initial denaturation at 95°C for 4 min followed by 40 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min in a PTC-200 DNA Engine Peltier Thermal Cycler (MJ Research, Inc., Watertown, MA). PCR amplicons were cloned, and sequenced, as indicated above.

Phylogenetic Analysis

SOO virus sequences from four Vero E6 cell-culture isolates were aligned and compared with previously published hantavirus sequences [Liang et al., 1994; Baek et al., 1998; Yashina et al., 2000, 2001], using the Clustal W method (Lasergene program version 5, DNASTAR, Inc., Madison, WI). The GenBank accession numbers for the S segment of SOO virus strains SOO-1, SOO-2, SOO-3, and SOO-4 were AY675349, AY675350, AY675351, and AY675352; for the M segment of strains SOO-1, SOO-2, and SOO-3, they were AY675353, DQ056293, and DQ056295; and for the L segment of strains SOO-1 and SOO-2, they were DQ056292 and AY675354, respectively. Phylogenetic trees were constructed by the neighbor-joining method, unweighted pair-group method of assortment (UPGMA), and maximum parsimony PAUP (Phylogenetic Analysis Using Parsimony, version 4). Topologies were evaluated by bootstrap analysis of 1,000 iterations [Swofford, 2003].

RESULTS

Serology and Virus Isolation

Using the IFA test, IgG antibodies against HTN virus were detected in 24% (6/25) of *A. peninsulae* captured on Mt. Gyebang and Mt. Gachil in Gangwon Province in August 1997, and in 35% (10/29) of *A. peninsulae* captured on Mt. Deogyu in Jeollabuk Province in September 1998 (Table I).

Characteristic hantavirus-specific fluorescence was detected by IFA in Vero E6 cells at 80 days after inoculation with 5% lung homogenates from hantavirus-seropositive *A. peninsulae*. The new hantavirus isolates in cell culture from *A. peninsulae*, designated SOO virus, were serially passed in Vero E6 cells. SOO virus strains SOO-1 and SOO-2 were isolated from *A. peninsulae* captured at Gangwon Province, Northeastern Korea, and strains SOO-3 and SOO-4 from Korean

TABLE I. Field Survey of Hantavirus Infection in Rodents at Mt. Gyebang at Mt. Gachil, in Hongcheon-gun, Gangwon Province, and Mt. Deogyu in Muju-gun, Jeollabuk Province, Republic of Korea, 1997–1998

Rodent species	Number of rodents captured	Number of seropositive (%)
Apodemus agrarius	25	2 (8.0)
Apodemus peninsulae	54	16 (29.6)
Eothenomys regulus	7	1 (14.3)
Micromys minutus	1	0 (0)

TABLE II. Antigenic Characterization by Cross-FRNT (80% reduction)

		Virus			
Antiserum	Animal	H5	HTN Bao14	HTN 76–118	SOO-3
H5[a]	Mouse	80	80	40	320
#61[b]	*A. peninsulae*	160	20	20	640
#63[b]	*A. peninsulae*	320	40	40	640
HTN Bao14	Mouse	40	1280	320	160
HTN 76–118	Mouse	<40	320	320	80

[a]Antisera against H5, HTN Bao14, and 76–118 were made from 5 weeks old ICR mice. Viruses (1×10^2–1.6×10^3 FFU) inoculated by subcutaneously and the mice were bled after 2 months. The underlined numbers are FRNT titers against homologus antisera and highest titer.
[b]Seropositive *A. peninsulae* were captured in the suburbs of Vladivostok, Russia.

field mice captured at Jeollabuk Province, South-Central Korea.

Antigenic Characterization by Monoclonal Antibodies

The antigenic characterization of HTN (strain 76–118), H5 and SOO (strain SOO-3) viruses was performed using a panel of MAb to glycoproteins G1, G2, and NP, which included two MAb to G1-1 (6D4 and 10F11), three to G1-b (16D2, 3D5, and 2D5), two to G2-a (HCO2 and 16E6), one to G2-b (EB06), one to G2-c (11E102-2), three to G2-d (17G6, 3D7, and 5B7), one to G2-e (20D3), six to G2-f (8E10, 23G10-1, 1C6, 7G6, 3B6, and 18F5), one to NP-1 (ECO2), and three to NP-III (C16D11, C24B4, and F23A1) [Arikawa et al., 1989; Lokugamage et al., 2004]. MAb 2D5 may recognize a specific epitope on G1-b of *A. agraius*-borne HTN virus, as evidenced by the strong reaction to HTN 76–118 and the absent reaction to H5 and SOO-3.

Focus-Reduction Neutralization Test

FRNT showed that SOO virus was antigenically distinct from *A. agrarius*-borne HTN virus 76–118 (Table II). The titers of anti-H5 serum and #61 and #63 sera against SOO virus strain SOO-3 were 8- to 32-fold higher than that against HTN virus 76–118. The titer of the anti-HTN virus 76–118 serum against HTN 76–118 was four-fold higher than that against SOO-3. These results suggest that *A. peninsulae*-borne SOO and H5 viruses are antigenically distinguishable from *A. agrarius*-borne HTN virus.

Sequence Analysis of Soochong Virus

The complete S segment, sequenced for the cell-culture isolates of SOO virus strains SOO-1, SOO-2, SOO-3, and SOO-4, was 1,695 nucleotides in length, with a predicted nucleocapsid protein of 430 amino acids starting at nucleotide position 37. Also, the SOO virus had a 371 nucleotide-length 3′ noncoding region (NCR). The intra-strain genetic divergence of the entire S segment of the Northeastern strains (SOO-1 and SOO-2) and South-central strains (SOO-3 and SOO-4) was 1.1% and 1.2% at the nucleotide level, respectively (Table III), whereas the divergence between the Northeastern and South-central strains was 12%–13% and 1.7%–2.9% at the nucleotide and amino acid levels, respectively. Six amino acid differences were observed between the SOO-1 and SOO-2 strains, and one amino acid differed between the SOO-3 and SOO-4 strains. Also six amino acids (positions 233, 241, 251, 271, 322, and 357) differed between the Northeastern and South-central strains. The entire S genomic segment of SOO virus SOO-1 strain diverged from HTN virus 76–118 strain by 15.6% and 3.5% at the nucleotide and amino acid levels, respectively.

TABLE III. Percent Nucleotide and Amino Acid Sequence Homologies of the Entire S, M, and L Segmenst Between Soochong Virus Strain SOO-1 and Other *Apodemus* Rodent-Borne Hantaviruses

	S segment		M segment		L segment	
Virus species/strain	1290 nt	430 aa	3615 nt	1135 aa	6533 nt	2151 aa
Soochong SOO-2	98.9	98.6	98.6	98.4	98.6	99.4
Soochong SOO-3	89.0	98.4	89.8	96.4	ND	ND
Soochong SOO-4	88.9	98.1	87.4	95.9	ND	ND
Soochong Liu	91.5	98.6	87.0	96.8	ND	ND
Amur H5	91.5	98.8	87.0	96.9	ND	ND
Amur SLAP61	91.2	99.8	ND	ND	ND	ND
Amur AP708	90.8[a]	99.5	ND	ND	ND	ND
Hantaan 76–118	84.4	96.5	80.7	91.1	81.4	95.6
Dobrava Greece	72.8	83.4	71.6	77.0	75.0	85.5

ND, not determined because of insufficient sequence data.
[a]Entire coding region of S segment.

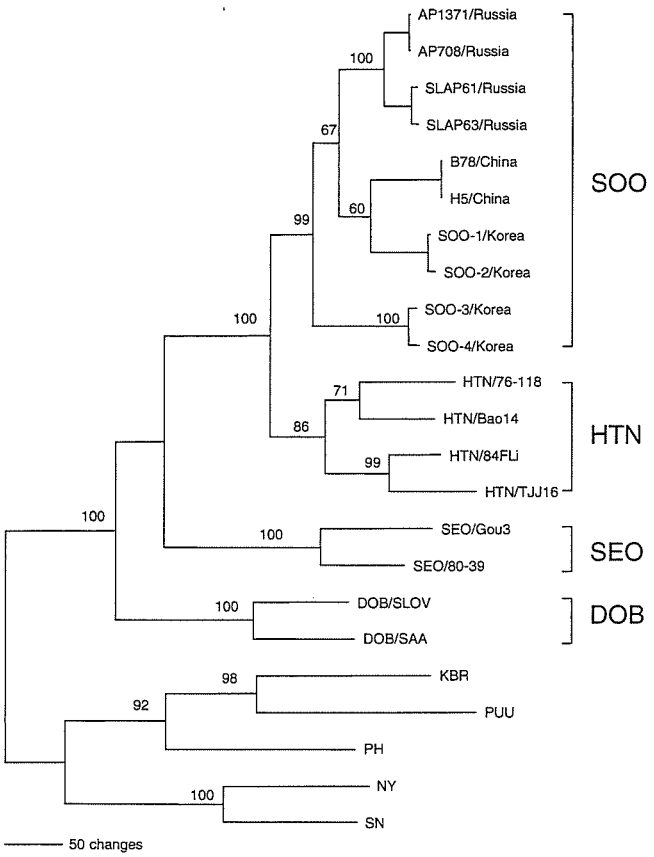

Fig. 2. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire coding region of the S segment of hantaviruses. The phylogenetic position of Soochong (SOO) virus strains is shown in relationship with hantavirus isolates from an HFRS patient in China (H5 and B78) and with Amur virus from *A. peninsulae* captured in Russia (SLAP61, SLAP63, AP708, and AP1317). Branch lengths are proportional to the number of nucleotide substitutions, while vertical distances are for clarity only. The numbers at each node are bootstrap probabilities (expressed as percentages), as determined for 1,000 iterations by PAUP version 4.0b.

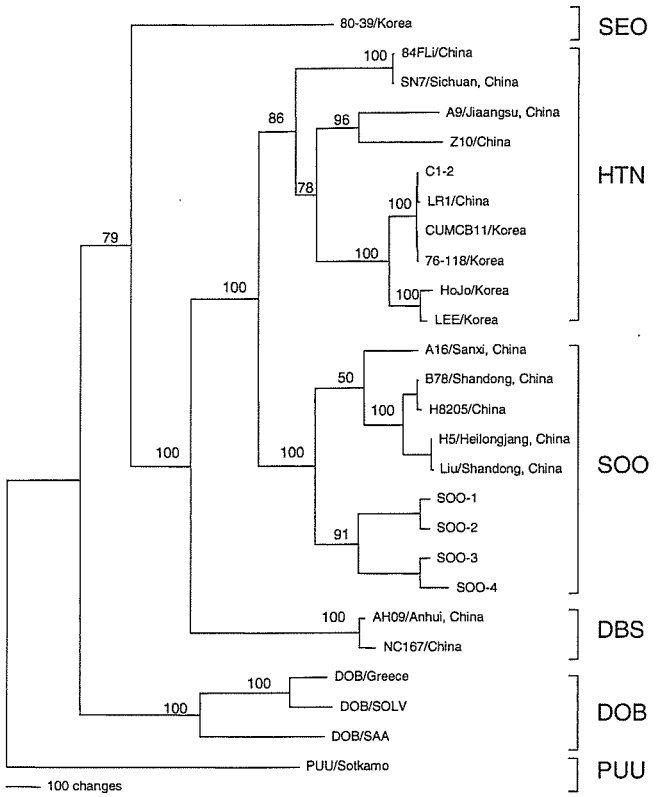

Fig. 3. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire M segment except 18-nucleotides of both ends of hantaviruses.

The full-length M genomic segment of SOO viruses was 3,615 nucleotides, with a predicted glycoprotein of 1,135 amino acids. Also, the SOO virus had 5′- and 3′-NCR of 40 and 166 nucleotides, respectively. The intra-strain genetic divergence of the entire M segment of Northeastern and South-central isolates was 1.5% and 3.2% at the nucleotide level, respectively, whereas the divergence between the Northeastern and South-central strains was 10.8%–14.1% and 3.5%–24.3% at the nucleotide and amino acid levels, respectively. The entire M genomic segment of SOO virus diverged from HTN virus 76–118 by about 22.6%–23.1% at the nucleotide level and 8.3%–9.5% at the amino acid level.

The entire L genomic segment of SOO viruses was 6,533 nucleotides, with a predicted coding capacity of 2,151 amino acids. The SOO 5′- and 3′-NCR was 37 and 39 nucleotides. The complete L genomic segment of SOO virus SOO-1 and SOO-2 strains diverged from HTN virus 76–118 by 21.8%–21.9% at the nucleotide level and 4.6% at the amino acid level.

Phylogenetic Analysis

Phylogenetic analyses of the nucleotide sequences of the S, M, and L segments of SOO virus and other hantaviruses, using the maximum parsimony and neighbor-joining methods, indicated that SOO virus was distinct from HTN virus and was more closely related to B78 and H5 viruses from China and Amur virus from Far East Russia (Figs. 2–4). Topologies were supported by bootstrap analysis of 1,000 iterations. A neighbor-joining tree, based on the 268-nucleotide partial M segment of hantaviruses (nucleotide position 2034–2301 compared to SOO-1 strain) showed that SOO virus shared a common ancestry with Amur virus from Russia and H5 virus from China, and formed a geographic-specific cluster of *A. peninsulae*-borne hantavirus strains (Fig. 5).

Phylogenetic analysis based on a 424-nucleotide cytochrome b region of mtDNA sequences showed that *A. peninsulae*, *A. agrarius*, and *A. flavicollis* were distinct species that co-evolved with their hantaviruses (Fig. 6).

DISCUSSION

The number of HFRS cases in Korea has decreased significantly since a hantavirus vaccine (Hantavax®) was approved for use by the Korean Food and Drug Administration in 1990 [Cho et al., 2002]. However,

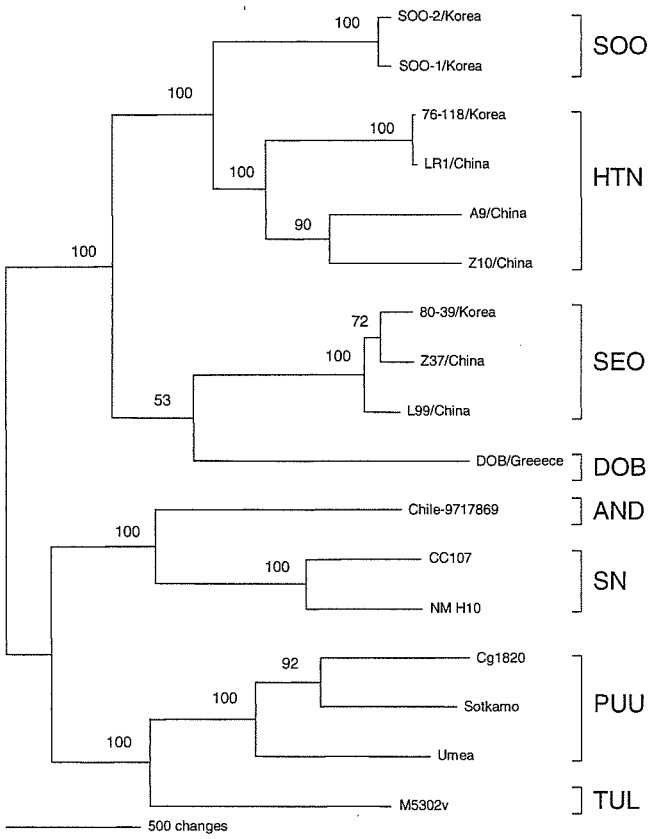

Fig. 4. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire L segment of hantaviruses.

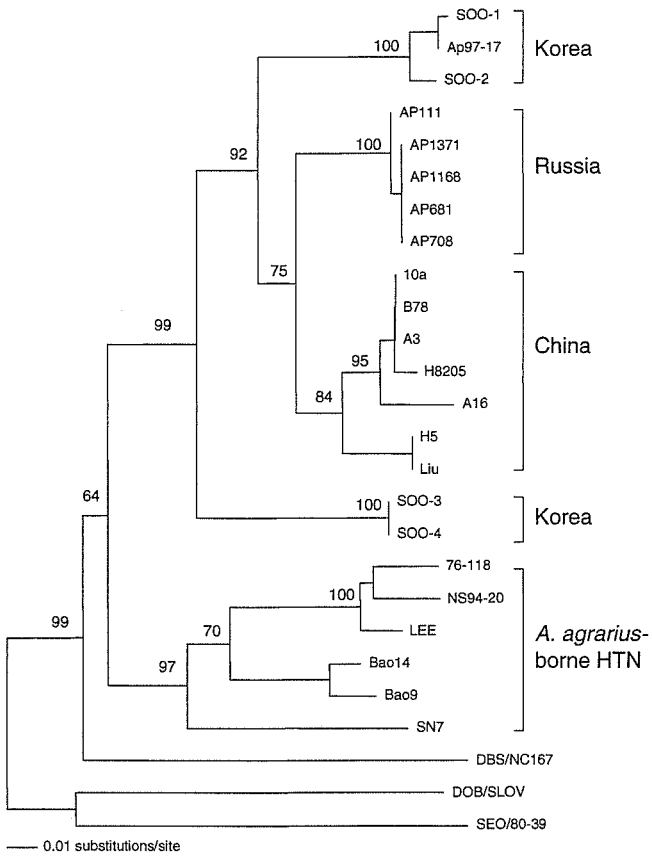

Fig. 5. Neighbor-joining trees, rooted at the midpoint, based on the 268-nucleotide partial M segment of hantaviruses (nucleotide position 2034–2301 compared to SOO-1 strain). Ap97-17 strain was amplified from *A. peninsulae* captured at Mt. Gyebang, Gangwon Province, Korea, 1997.

other factors, including ecological and environmental changes may also be responsible for this declining trend of HFRS. A recent case-control study reported that the effectiveness of Hantavax® depended principally on the number of doses received (with protection rates of 25%, 46%, and 75% for one, two and three doses, respectively) in Korea [Park et al., 2004]. Despite these epidemiological trends, HFRS continues to be regarded as one of the principal acute febrile diseases during the autumn season among military personnel and civilians in Korea [Sachar et al., 2003]. Of the approximately 100–300 HFRS cases (with 4.5% overall mortality) occurring annually in Korea, HTN virus is the primary etiologic agent, accounting for 70% of all cases, with 20% being attributed to SEO virus, and the remaining 10% to as yet undefined hantaviruses.

Each genetically distinct hantavirus appears to have co-evolved with one or a few closely related rodent species. Phylogenetic clustering of murid rodent-borne hantaviruses and their rodent reservoir hosts lend further support to the concept that hantaviruses co-evolved with rodents. *Apodemus* mice are the most common rodents inhabiting woodlands, tall grasses, rice paddies, and broadleaf forests in the temperate zone of the Palearctic region. Two or more species may be sympatric, or coexist in the same forest (e.g., *A. agrarius* and *A. peninsulae* in Korea). The Korean field mouse (*A. peninsulae*) has a wide geographic distribution, extending from eastern and southern Siberia, Manchuria, northeastern and central China, Korea, and as far easterly as Sakhalin and Hokkaido [Nowak, 1999]. This is the second-most dominant rodent species in Korea, and is primarily found in forested mountainous areas >500 m in elevation.

Four strains of SOO virus, SOO-1, SOO-2, SOO-3, and SOO-4, were isolated in Vero E6 cells from lung tissues of *A. peninsulae* captured in two geographically distant provinces at separate times. These SOO virus strains are the first hantaviruses isolated in cell culture from *A. peninsulae*. Although genetically similar hantaviruses, H5 and B78, have been isolated from HFRS patients in China [Liang et al., 1994], no report is currently available on the isolation of Amur virus from *A. peninsulae* in Russia. The antigenic characterization by a panel of MAb to glycoproteins G1, G2, and NP showed that MAb 2D5, which recognizes an epitope on the G1-b region, could distinguish the *A. peninsulae*-borne SOO hantavirus from the *A. agrarius*-borne HTN virus. Also, MAb 16D2 of the G1-1b region reacted with HTN 76–118 and H5 viruses, but not with SOO virus, suggesting that there is a distinct epitope between SOO

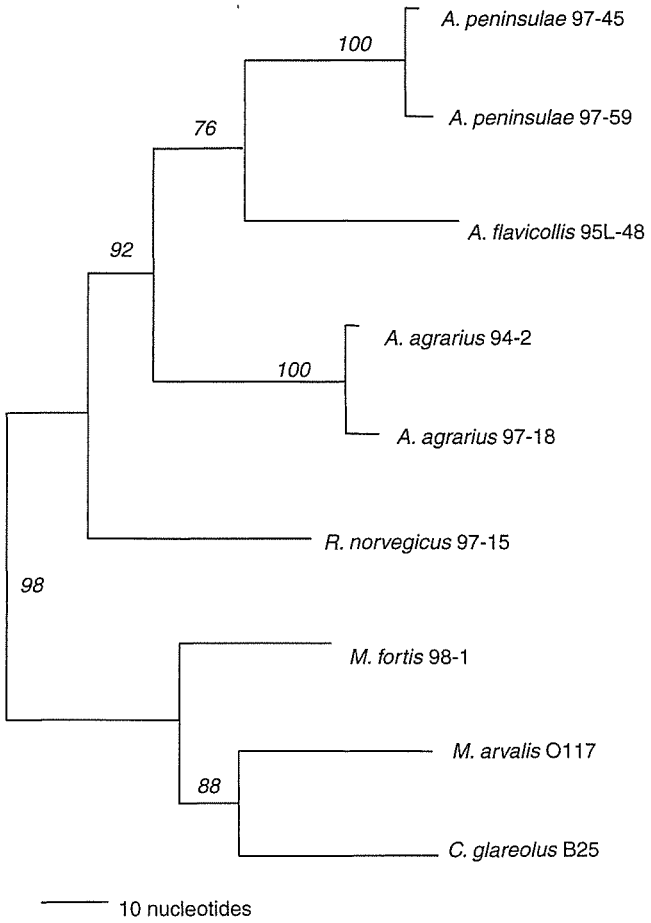

Fig. 6. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on a 424-nucleotide region of the cytochrome b gene of mtDNA from reservoir rodent species of hantaviruses. mtDNA sequences were amplified from liver tissues of *A. peninsulae* 97-45 (SOO-1) and 97-59 (SOO-2), *A. agrarius* 94-2 and 97-18, *R. norvegicus* 97-15 and *M. fortis* 98-1 captured in Korea; *A. flavicollis* 95-L48 and *M. arvalis* O117 captured in Poland; and *C. glareolus* B25 from Sweden.

and H5. The cross-neutralization test using anti-HTN 76–118 serum showed that the titer to HTN 76–118 and Bao14 were 320. However, the NT titer to SOO-3 and H5 were 80 and <40, respectively. These cross-NT results and previously reported M segment sequences suggest that *A. peninsulae*-borne SOO and Amur viruses are antigenically distinguishable from *A. agrarius*-borne HTN virus [Lokugamage et al., 2004].

In our previous report on the genetic diversity of HTN virus in Korea, H5 was an outgroup of all other Korean and Chinese HTN virus strains [Song et al., 2000]. Phylogenetically, the Northeastern SOO virus strains (SOO-1 and SOO-2) were distinguishable from the South-central SOO virus strains (SOO-3 and SOO-4) in Korea. SOO virus isolated from the Korean field mouse in Korea shared a common ancestry with Amur virus strains, including SLAP61, SLAP63, AP708, and AP1371 from *A. peninsulae* of Far East Russia, as well as with H5 and B78 viruses from HFRS patients in Heilongjiang and Shandong, China [Liang et al., 1994; Wang et al., 2000; Lokugamage et al., 2002]. Thus, *A. peninsulae*-borne hantaviruses were evolutionarily distinct from HTN and DOB viruses harbored by *A. agrarius* and *A. flavicollis*, respectively. The high seropositivity rates among *A. peninsulae* captured in certain mountainous regions in Korea suggest that HFRS caused by SOO virus may be under-reported among nearby residents or among individuals who might visit such areas for recreational or occupational purposes.

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Development of Serological Assays for Thottapalayam Virus, an Insectivore-Borne Hantavirus[▽]

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Thottapalayam virus (TPMV), a member of the genus *Hantavirus* in the family *Bunyaviridae*, was isolated from an insectivore, *Suncus murinus* (musk shrew), captured in southern India in 1964. While the isolation of TPMV predates the discovery of the prototype Hantaan virus, little is known about its genetics and biology. To date, preliminary evidence suggests that TPMV differs significantly, both antigenically and genetically, from all known rodent-borne hantaviruses. However, since detailed epizootiological studies have not been conducted, it is unclear if TPMV is naturally harbored by an insectivore host or if TPMV represents a "spillover" from its natural rodent reservoir host. Moreover, to what extent TPMV causes infection and/or disease in humans is not known. To address these issues, we first studied the antigenic profile of TPMV using monoclonal antibodies against Hantaan and Seoul viruses and polyclonal immune sera against Puumala virus and TPMV. Armed with this newfound information, we developed an enzyme-linked immunosorbent assay system for the diagnosis of TPMV infections in shrews and humans, using a recombinant TPMV N antigen manipulated to have an E5/G6 epitope to be captured by monoclonal antibody clone E5/G6. Using this assay, we found anti-TPMV antibodies in sera from a patient with high fever of unknown etiology in Thailand and from two shrews captured in Indonesia. Seropositivity was verified by the indirect immunofluorescence antibody test, Western blotting analysis, and focus reduction neutralization test. Collectively, our data indicate that TPMV is harbored by *Suncus murinus* as its host in nature and is capable of infecting humans.

Fn1 Like other viruses in the family *Bunyaviridae*, members of the genus *Hantavirus* are enveloped viruses with a tripartite, negative-stranded RNA genome, consisting of large (L), medium (M), and small (S) segments. The L segment encodes an RNA-dependent RNA polymerase; the M segment encodes a glycoprotein precursor, which is cleaved into surface glycoproteins, Gn and Gc; and the S segment encodes a nucleocapsid protein (N) (15). Some hantaviruses cause zoonotic diseases in humans, known as hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome (HPS) (14). Presently, 22 species are classified within the genus *Hantavirus* based on antigenic and genetic differences (9). In the Old World, four antigenically related and genetically distinct hantaviruses are known to cause hemorrhagic fever with renal syndrome: Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), and Dobrava virus (DOBV). Several sigmodontine rodent-borne hantaviruses in the New World, including Sin Nombre virus (SNV) and Andes virus, cause HPS. For both diseases, virus transmission to humans occurs via aerosolization of infectious rodent excreta (6).

Each hantavirus appears to have coevolved with a specific rodent species, in which it maintains an enzootic cycle. As the only known presumed exception, Thottapalayam virus (TPMV) was isolated from an insectivore, *Suncus murinus*, captured in southern India in 1964 (3). Either very low or no antigenic cross-reactivity has been observed between TPMV and other hantaviruses (4, 5). And as evidenced by nucleotide and amino acid sequence analyses of the full-length S segment, TPMV is the most genetically divergent of all other hantaviruses (6, 17). Analyses of the recently acquired full-length M and L segments of TPMV are congruent (J.-W. Song and R. Yanagihara, unpublished observations). However, since detailed epizootiological and epidemiological surveys of TPMV infection have not been conducted, the fundamental biology of TPMV, including its true natural host and pathogenicity to humans, is unclear.

Previously we developed enzyme immunoassays using baculovirus-expressed recombinant N (rN) antigens of various hantaviruses (including HTNV, SEOV, PUUV, and DOBV) for the serological diagnosis of hantavirus infections (1, 7, 8, 18). With this method, the monoclonal antibody (MAb) clone

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TABLE 1. Reactivities of MAbs to TPMV and rodent-borne hantaviruses

Specificity	Epitope	MAb	Reactivity of MAb to[a,b]:								
			TPMV	HTNV	SEOV	THAIV	DOBV	SaaV	PUUV	PHV	SNV
N (HTNV)		FDO3	±	+	+	+	+	+	+	+	NT
		KAO6	−	+	+	+	+	+	−	−	NT
		ECO2	−	+	+	+	+	+	−	−	−
		ECO1	−	+	+	+	+	+	+	−	−
		GBO4	−	+	+	+	+	+	+	+	NT
		E5G6	−	+	+	+	+	+	+	+	+
		C16D11	−	+	+	+	+	+	+	+	−
		F23A1	−	+	+	+	+	+	−	+	+
		C24B4	−	+	−	−	±	±	−	−	−
		BDO1	−	+	−	−	−	−	−	−	−
		G5	−	+	−	−	−	−	−	−	+
N (SEOV)		DCO3	−	−	+	−	−	−	−	−	−
Gn/Gc (HTNV)	Gn-a	8B6	−	+	±	+	±	+	±	−	NT
		6D4	−	+	+	+	+	+	−	−	NT
		10F11	−	+	+	+	+	+	+	−	NT
	Gn-b	2D5	−	+	−	−	−	−	−	−	NT
		3D5	−	+	−	−	−	−	−	−	NT
		16D2	−	+	−	−	−	−	−	−	NT
	Gc-a	HCO2	−	+	+	+	−	−	−	−	NT
		16E6	±	+	+	+	+	+	±	−	NT
	Gc-b	EBO6	−	+	+	−	±	±	−	−	NT
	Gc-c	11E10	−	+	±	−	+	−	+	+	NT
	Gc-e	17G6	+	+	±	+	+	+	+	±	NT
		3D7	+	+	+	+	+	+	+	±	NT
		5B7	+	+	+	+	+	+	+	±	NT
	Gc-e	20D3	+	+	±	+	+	+	−	−	NT
	Gc-f1	8E10	+	+	+	+	+	+	+	±	NT
		1C6	+	+	+	+	+	+	+	±	NT
		1G8	−	+	+	+	+	+	+	±	NT
		23G10-2	+	+	+	+	+	+	+	±	NT
		3B6	±	+	+	+	+	+	+	±	NT
	Gc-f2	23G10-1	+	+	+	+	+	+	−	−	NT
		7G6	−	+	+	+	+	+	−	−	NT
		18F5	−	+	±	+	+	+	−	−	NT

[a] TPMV, Thottapalayam virus; HTNV, Hantaan virus; SEOV, Seoul virus; THAIV, Thailand virus; DOBV, Dobrava-Belgrade virus; SaaV, Saaremaa strain of DOBV; PUUV, Puumala virus; PHV, Prospect Hill virus; SNV, Sin Nombre virus; NT, not tested.
[b] Results with HTNV, SEOV, PUUV and PHV (2), THAIV, DOBV, and SaaV (12), and SNV (7) are available in previous reports.

E5/G6 is utilized as an effective capture antibody, since it binds to a linear epitope of the N protein among all hantaviruses (11, 18). Thus, after determining the antigenic profile of TPMV, we developed a robust serological assay to diagnose TPMV infections in animals and humans, using the TPMV rN antigen manipulated to contain specific amino acid substitutions to allow binding with MAb E5/G6. Using this assay, we detected anti-TPMV antibodies in a human with febrile illness and in two musk shrews. These results indicate that TPMV is carried by musk shrews in nature and is capable of causing infections in humans.

MATERIALS AND METHODS

Viruses and cells. The prototype VRC-66412 strain of TPMV, originally isolated in suckling mice (3) and subsequently adapted to growth in the E6 clone of Vero cells (CRL 1586; American Type Culture Collection), was used. HTNV strain 76-118, SEOV strain SR-11, and PUUV strain CG1820 were used as representative rodent-borne hantaviruses. Viruses were propagated in Vero E6 cells maintained in Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% nonessential amino acids (Gibco). High Five cells (Invitrogen, Carlsbad, CA) were maintained in Grace's insect growth medium (Gibco) supplemented with 10% fetal bovine serum. Recombinant baculoviruses of HTNV, PUUV, and SNV were prepared as described previously (1).

MAbs and immune sera. Monoclonal antibodies (MAbs) and immune rabbit sera for N of HTNV and SEOV and MAbs to Gn and Gc of HTNV, as described previously, were used (2, 18). Immune rabbit serum for PUUV N was kindly provided by Hiroaki Kariwa of the Graduate School of Veterinary Medicine, Hokkaido University. Immune rabbit serum to TPMV N was prepared by intradermal injections of an 11-week-old Std:JW/CSK rabbit (specific-pathogen-free rabbit; SLC, Shizuoka, Japan) with 350 μg of TPMV rN expressed in *Escherichia coli* and 500 μg of Freund's complete adjuvant. A booster immunization of the same antigen with Freund's incomplete adjuvant was administered at 24 days, and blood was collected at 58 days. Immune mouse sera to TPMV were obtained 4 weeks following intraperitoneal inoculation of BALB/c mice (CLEA Japan, Osaka, Japan) with 2.0×10^3 focus-forming units of native TPMV (indirect immunofluorescence antibody [IFA] titer against TPMV was 1:12,800). Finally, sera were obtained from shrews (CLEA Japan) inoculated subcutaneously with 5.2×10^4 focus-forming units of native TPMV at 40 days postinoculation.

Human patient and wild shrew sera. Of the 478 human sera available for testing, 284 were collected between 2003 and 2004 from patients in Surin Province who had leptospirosis-like symptoms but who were serologically negative for both *Leptospira* and dengue virus. The other 194 sera were collected from patients with febrile illnesses of unknown etiology as part of the Emerging Infectious Diseases collaborative project, conducted by the Thai National Institute of Health and the Japan International Cooperation Agency in Nongkhai Province in 2005. In addition, sera were collected from 14 wild shrews

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(*Suncus murinus*) captured in Thousand Islands, Indonesia, in July and October 2005.

Preparation of recombinant TPMV N antigen. Culture supernatant of TPMV-infected Vero E6 cells was ultracentrifuged (265,000 × *g*, 4 h, 4°C), and RNA was isolated from the viral pellet and dissolved with Isogen (Invitrogen) following the manufacturer's instructions. Reverse transcription-PCR was performed using the KOD-plus system (Toyobo, Tokyo, Japan) to amplify the entire TPMV N-coding S segment with primers 5′-TTCAG *AATTC* GATGA CTCAA GGGAA AATGA CTCCC GAAGA-3′ and 5′-TATCC *TCGAG* TTACA GTTTA ATAGG CTCCT GACTT GAAAT C-3′ (the EcoRI and XhoI sites are shown in italics). After amplification, the DNA fractions were subcloned into the pET-43b(+) vector using restriction enzymes that recognized the restriction sites added by PCR and transformed into *E. coli* strain Origami (Invitrogen). A single colony was inoculated into Circle growth medium (BIO101 systems, Carlsbad, CA) containing tetracycline, kanamycin, and ampicillin for small-scale culture incubation at 37°C overnight. The culture fluid was then centrifuged, the collected cells were inoculated into 100 ml of fresh medium, and isopropyl-β-D-thiogalactopyranoside induction was performed according to the procedure for pET system expression. The cultured cells were collected by centrifugation, resuspended in 5 ml of 0.5 M NaCl binding buffer (0.5 M NaCl, 20 mM imidazole, 20 mM potassium phosphate), and sonicated four times for 15 s each on ice. Thereafter, the fusion protein was purified using a His trap column (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

IFA test. An IFA test was performed using previously described procedures (18). Acetone-fixed smears of Vero E6 cells infected with hantavirus or High Five cells infected with recombinant baculovirus were used as antigens. Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) (heavy plus light chains) antibody (1:2,000; Molecular Probes, Eugene, OR) was used as a secondary antibody to MAbs. For rabbit and human sera, fluorescein isothiocyanate-conjugated protein A (1:2,000; Sigma, St. Louis, MO) was used. IFA titers were expressed as the reciprocal of the highest serum dilution that produced characteristic intracytoplasmic fluorescence.

Peptide synthesis and antigenic analysis. Peptides were synthesized and analyzed by previously published methods (11). Briefly, using an Autospot ASP222 peptide synthesizer (ABiMED, Langenfeld, Germany), a variety of 10-mer peptides were spotted on a membrane. The spotting membrane was blocked in Block Ace (Yukijirushi Co., Tokyo, Japan) for 30 min at room temperature, stained with an E5/G6 hybridoma culture supernatant for 60 min, and detected using horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:500; Zymed, South San Francisco, CA) and 3-amino-9-ethylcarbazole (Sigma).

Construction of recombinant baculovirus expressing TPMV N with an E5/G6 epitope. The subcloned DNA fragment was excised from pET-43(+), described above, by digestion with the same enzyme and inserted in the donor plasmid pFAST-BAC1 (Gibco). Based on the results of the E5/G6 epitope analysis of TPMV N, amino acid-altering nucleotide mutations required for E5/G6 binding were added, using the GeneTailor site-directed mutagenesis system (Invitrogen). TPMV wild-type rN (rN/wt) and TPMV rN with the E5/G6 epitope (rN/E5G6) were expressed using the Bac-to-Bac baculovirus expression system according to the manufacturer's instructions (Gibco). These baculoviruses were inoculated into High Five cells to acquire the rN antigen, using previously described methods (1).

Western blotting analysis. Western blotting was performed using previously published methods (19). The infected High Five and Vero E6 cells were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (ATTO, Tokyo, Japan). Immune rabbit serum to hantavirus N was used to detect antigen on the membrane. Binding antibodies were detected using HRP-conjugated protein A (Prozyme, San Leandro, CA), and 4-chloro-1-naphthol (Sigma) was used as the peroxidase substrate.

ELISA. Enzyme-linked immunosorbent assays (ELISAs) using whole HTNV, PUUV, SNV, and TPMV rN were performed according to previously described methods (1, 7, 8, 18). MAb E5/G6 (2 μg/ml) was used as a capture antibody to coat 96-well plates for 60 min at 37°C. Nonspecific binding was blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS). After a 60-min incubation, the plates were washed three times with PBS containing 0.05% Tween 20. Each antigen was added and incubated for 60 min at 37°C, followed by three washings. For detection of rabbit IgG, HRP-conjugated goat anti-rabbit IgG antibody (1:5,000; Jackson, Bar Harbor, ME) was used as the secondary antibody, and *o*-phenylenediamine (Sigma) was added as the peroxidase substrate. Absorbance at 450 nm was measured using a SpectraMax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). For detection of human IgG, alkaline phosphatase-conjugated goat anti-human IgG (γ-chain specific) antibody (1:2,000; Sigma) was used as the secondary antibody and *p*-nitrophenyl phosphate (Sigma) was added as the substrate before measuring the absorbance at 405 nm. For detection of shrew IgG, HRP-conjugated protein A (1:5,000; Prozyme) was used as the secondary antibody and *o*-phenylenediamine was added as the peroxidase substrate.

FRNT. Endpoint titers of neutralizing antibodies were determined by the focus reduction neutralization test (FRNT), as described elsewhere (1). Foci of virus-infected cells were detected by staining the cells with Alexa Fluor 488-labeled MAb 5B7, which recognizes the Gc of hantaviruses (10). FRNT titers were expressed as the reciprocal of the highest serum dilution leading to a greater than 80% reduction in the number of infected cell foci.

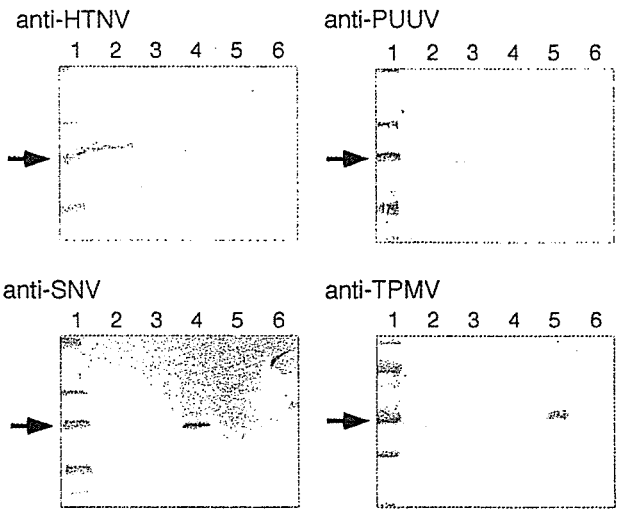

FIG. 1. Western blot analysis of hantavirus antigens using polyclonal rabbit immune sera. We tested the cross-reactivity of each hantavirus, including TPMV, using sera from rabbits immunized with rN antigens expressed in *E. coli*. For HTNV, PUUV, and TPMV antigens, viruses were inoculated on Vero E6 cells, harvested, dissolved, and used. For SNV antigen, High Five cells expressing SNV rN by use of recombinant baculovirus were used (7). Lanes 1, molecular weight marker; lanes 2, HTNV; lanes 3, PUUV; lanes 4, SNV; lanes 5, TPMV; lanes 6, uninfected Vero E6 cells. Arrows indicate the band at 50 kDa.

RESULTS

Antigenic profiling of TPMV using MAbs and polyclonal antibodies. To characterize the TPMV antigenic profile, we performed the IFA test using a panel of MAbs against HTNV N, Gc, and Gn and SEOV N (Table 1). None of the MAbs against HTNV N and Gn cross-reacted with TPMV, while 8 of 16 MAbs against HTNV Gc did. By contrast, all other hantaviruses exhibited various degrees of cross-reactivity to MAbs against N and Gn, except for Prospect Hill virus, which did not react with MAbs against HTNV Gn. TPMV seemed to share partly common epitopes in the Gc region but not in the N or Gn regions, although all other serotyped viruses had some common epitopes in each region. T1/AQ:B AQ: C

We next immunized a rabbit with TPMV rN expressed in *E. coli* and obtained a polyclonal immune serum with an IFA titer of 1:6,400, which strongly reacted also to reduced-TPMV antigens by Western blot analysis (Fig. 1). Using this immune serum, however, TPMV did not cross-react with other hantaviruses (Fig. 1), suggesting that TPMV was the most antigenically divergent of all hantaviruses isolated to date. F1

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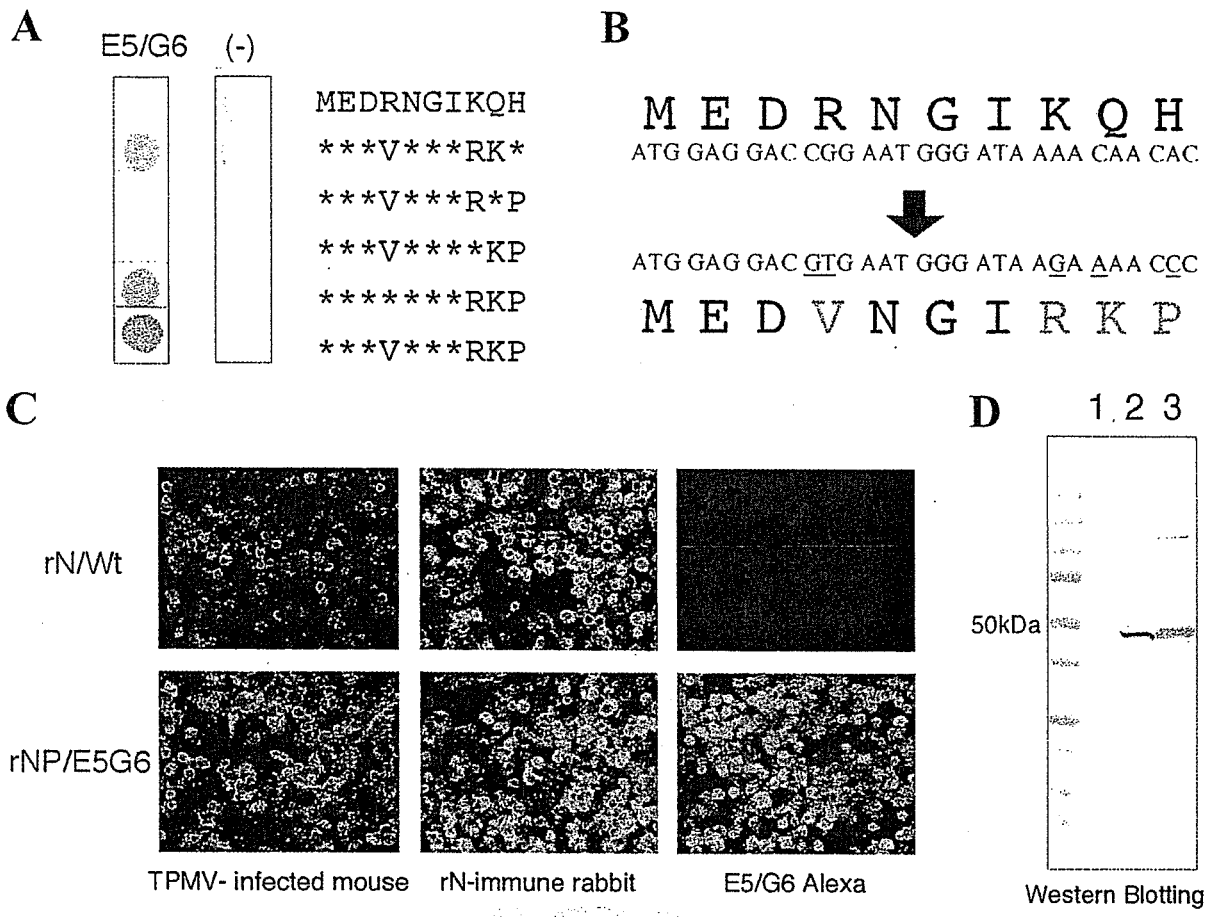

FIG. 2. A. E5/G6 epitope analysis of TPMV N. Using a variety of synthesized 10-mer peptides, we confirmed E5/G6 reactivity against TPMV sequence. Further, we determined which amino acid changes in this region were essential for E5/G6 binding. The peptide changes at positions 178 (R→V), 182 (K→R), 183 (Q→K), and 184 (H→P) were sufficient for MAb E5/G6 binding. B. Insertion of several amino acid mutations changing E5/G6 binding. C. Confirmation of the antigenicitiy of each baculovirus-infected High Five cell antigens expressed by recombinant baculoviruses. The rN antigen having the original sequence (rN/wt) reacted with immune serum but not with MAb E5/G6. On the other hand, the rN with the E5/G6 epitope (rN/E5G6) reacted with immune serum, as well as MAb E5/G6. D. Western blot analysis using sera from rabbits immunized with *E. coli*-expressed rN antigens. Both rN antigens (rN/wt and rN/E5G6) were detected by a band of about 50 kDa, which corresponded to authentic TPMV N. Lane 1, rN/wt; lane 2, rN/E5G6; lane 3, TPMV-infected Vero E6 cells.

Epitope analysis and construction of TPMV rN possessing the E5/G6 epitope. We have developed an ELISA system for diagnosing hantavirus infections with excellent specificity and sensitivity (1, 7, 8, 18). In this assay, baculovirus–expressed recombinant hantavirus antigen is captured on 96-well plates coated with MAb E5/G6 (18). Although there are several amino acid variations, the N protein of all 21 rodent-borne hantavirus species reacted with MAb E5/G6 (11). Therefore, MAb E5/G6 is an effective tool for capturing hantavirus N, with the only exception being TPMV (Table 1). Accordingly, to use this assay for TPMV, we manipulated the E5/G6 epitope region of TPMV rN to allow binding with MAb E5/G6. The MAb E5/G6 made by immunizing mice with HTNV rN reacted effectively with the sequence YEDVNGIRKP at 165 to 174 amino acids (11, 18). However, TPMV has the sequence ME DRNGIKQH for the corresponding E5/G6 epitope region and did not react with MAb E5/G6 (Table 1). Using a peptide synthesizer, we synthesized this 10-mer peptide and confirmed the effect of some amino acid mutations on MAb E5/G6. As a result, the peptide MEDVNGIRKP with four changes (R to V, K to R, Q to K, and H to P) reacted with MAb E5/G6 (Fig. 2A). Based on the E5/G6 epitope analysis, we inserted five nucleotide mutations in the TPMV S segment to produce the four amino acid changes (Fig. 2B) and prepared recombinant baculoviruses expressing TPMV rN with the E5/G6 epitope. F2

High Five cells inoculated with the recombinant baculoviruses were harvested, and the antigenicities of TPMV rN/wt and TPMV rN/E5G6 was confirmed by the IFA test (Fig. 2C). Both rNs reacted with TPMV-infected mouse sera and rN-immune rabbit sera. But only rN/E5G6 reacted with MAb E5/G6, as expected. In addition, we confirmed the antigenicity of rN/wt and rN/E5G6 by Western blotting analysis using rN-immune rabbit sera (Fig. 2D). These data show that both TPMV rNs have the same band of approximately 50 kDa, which is the size of TPMV N, as well as TPMV-infected Vero