表 2. 北海道の野生齧歯類におけるハンタウイルス抗体の保有状況

種	各調査地点における抗体陽性率，陽性数 / 検体数 (%)										
	佐呂間	小清水	津別	北見	根室	富良野	当別	野幌	苫小牧	上磯	合計
C. rufocanus	2/14 (14.3)	4/15 (26.7)	1/33 (3.0)	1/3 (33.3)	—	0/3 (0)	13/120 (10.8)	3/60 (5.0)	—	5/30 (16.7)	29/278 (10.4)
C. rutilus	—	—	—	—	0/4 (0)	—	—	—	—	—	0/4 (0)
A. speciosus	0/2 (0)	0/1 (0)	0/11 (0)	0/3 (0)	0/42 (0)	0/93 (0)	0/3 (0)	0/24 (0)	0/11 (0)	0/27 (0)	0/217 (0)
A. argenteus	0/24 (0)	0/5 (0)	0/10 (0)	0/26 (0)	—	0/30 (0)	—	—	0/3 (0)	0/66 (0)	0/164 (0)
合計	2/40 (5.0)	4/21 (19.0)	1/54 (1.9)	1/32 (3.1)	0/46 (0)	0/126 (0)	13/123 (10.6)	3/84 (3.6)	0/14 (0)	5/123 (4.1)	663 (4.4)

より，原因ウイルスはシカシロアシマウスという北アメリカ大陸に固有の齧歯類が病原巣動物であることが明らかになり[12]，シンノンブレウイルスと名付けられた。さらに，ウイルスは以前からシカシロアシマウスに保有されており，古くからHPSの散発的発生があったことも確認された。1993年のHPSの多発は地球規模の気象変動が原因だったと考えられている。1992年から1993年にかけて発生したエルニーニョ現象による降雨量の増加のため，北米大陸の南西部の砂漠地帯が緑地化したことが知られている。これにより，齧歯類の爆発的な繁殖が起こり，人と感染齧歯類との接触機会が増加したために，HPSが多発したものと考えられる。このように，環境の変化によって人獣共通感染症の発生状況が激変することがあるため，自然界における病原体の存続や伝播の様式を事前に解明しておくことが重要である。1993年の流行後も継続的なHPSの発生が見られており，2004年3月までに米国だけで363名の患者が報告されている。また，シカシロアシマウスの他にも北米大陸でHPSを媒介する齧歯類が複数存在することが明らかになり，これらの齧歯類がシンノンブレウイルスに近縁ではあるものの異なったハンタウイルスを保有していることも次第に明らかにされた。米国以外のアメリカ大陸でもHPSの発生が相次いでいる。2002年までにカナダ，アルゼンチン，チリ，パラグアイ，ウルグアイ，ブラジル，ボリビアなどから合計1,254名の患者が報告されている。HPSもHFRSと同様に感染齧歯類の排泄物を吸い込むことによって感染が起こるが，HFRSと同様に人から人への感染は起こらないと考えられていた。しかし，1996年アルゼンチンで発生した流行では人から人への空気感染が起こったことが判明した[13]。しかし，人から人へのHPSの感染は非常にまれなことと考えられている。

3．予防方法

ハンタウイルス感染症は齧歯類によって媒介されることから，まず齧歯類集団において抗体調査を行って，流行地と病原巣動物を特定することが予防対策上重要である。流行地では齧歯類を人に近づけないことが最大の対策となる。すなわち，ネズミの駆除や衛生的な環境整備（ネズミの餌となるようなものを長期間保存しない，残飯などを放置しない）などを心懸けるべきである。

わが国ではハンタウイルス感染症に対するワクチンは開発されていない。

4．わが国および近隣諸国の齧歯類におけるハンタウイルス感染症の疫学調査

わが国では人の感染例が極めて稀であるため，本症は外来性感染症でウイルス自体が日本に存在しないかのように錯覚されやすい。しかし，ドブネズミやエゾヤチネズミはウイルスを今も保持し続けている[8,9]。ドブネズミの保有するウイルスは，かつて日本各地の実験動物施設でHFRSの原因となったソウルウイルスに属している[14]。北海道では広い範囲でエゾヤチネズミが本ウイルスに感染している（表2）[9]。本ウイルスはヨーロッパでHFRSの原因となっているプーマラウイルスに近縁であることが判明しているものの[15]，ウイルス分離が困難なことから，これまでその詳細な性状は不明であった。現在，当教室で本ウイルスの分離法や検出法の改良が進められつつあるので，今後エゾヤチネズミの保有するウイルスの性状解析が進むものと期待される。また，本ウイルスの人への感染の有無について，今後調査する必要がある。また，全国規模の野生齧歯類の疫学調査を行ったところ，ドブネズミやエゾヤチ

表3　日本の齧歯類と食虫類におけるハンタウイルス抗体の保有状況

種	抗体陽性例	捕獲数	抗体陽性率 (%)
A. speciosus	5	482	5/482 (1.0)
C. rufocanus	7	197	7/197 (3.6)
R. norvegicus	4	364	4/364 (1.1)
R. rattus	3	45	3/45 (6.7)
A. argentius	0	59	0/59 (0)
A. peninsule	0	4	0/4 (0)
C. rutilus	0	22	0/22 (0)
E. smithi	0	11	0/11 (0)
M. minitus	0	1	0/1 (0)
M. montebelli	0	11	0/11 (0)
M. musculus	0	4	0/4 (0)
C. dsinezumi	0	1	0/1 (0)
S. caecutiens	0	3	0/3 (0)
S. gracillium	0	11	0/11 (0)
S. nguiculatus	0	1	0/1 (0)
U. tapoides	0	5	0/5 (0)
合計	19	1221	19/1221 (1.6)

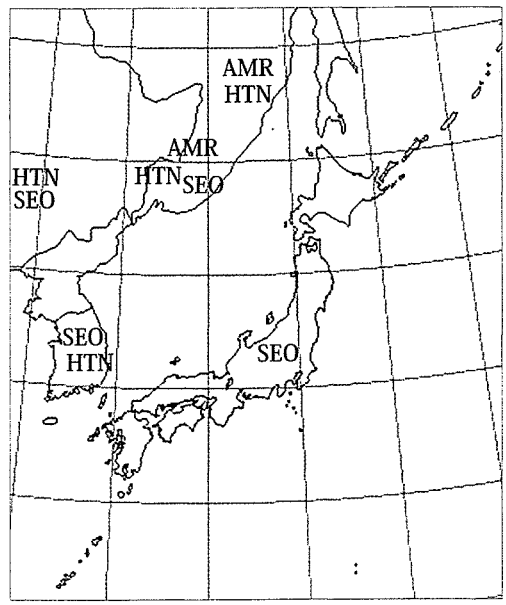

図2　東アジアにおける病原性ハンタウイルスの分布
極東ロシア，中国および韓国では重症型 HFRS の原因ウイルスであるハンターンウイルスとアムールウイルスが分布している。中等度の病原性を示すソウルウイルスは大陸と日本に分布している。
HTN：ハンターンウイルス
AMR：アムールウイルス
SEO：ソウルウイルス

ネズミの他にも，クマネズミとアカネズミでハンタウイルス抗体が検出されることが明らかになった(表3)。さらに，わが国の人においても低率ではあるが本ウイルスに対する抗体が検出されている。したがって，ハンタウイルスの感染はわが国においても発生していることが示唆される。

日本の近隣諸国ではわが国と異なり，強毒型のハンタウイルスが分布しており，毎年多数の HFRS 患者が発生している。極東ロシアや中国ではハンターンウイルスの他にも人に重篤な HFRS を引き起こすアムールウイルスと呼ばれるハンタウイルスが存在し，本ウイルスがハントウアカネズミを病原巣動物として存在することが遺伝子解析の結果から明らかになった（図2，3）[16]。したがって，これらの地域で野外活動する予定の旅行者は HFRS に感染する可能性がある。日本国内には HPS を媒介する齧歯類が生息していないことから，幸いこれまでに HPS の患者発生は認められていない。しかし，南北アメリカ大陸には HPS の媒介動物が広く分布していることに注意を払う必要がある。

5．外国産野生齧歯類の輸入規制強化

財務省の貿易統計によれば平成14年には約75万匹の齧歯類が我が国に輸入されていた。このうち人獣共通感染症を媒介する可能性のある野生齧歯類は推定で約5万匹ほどであったと考えられる。これまではラッサ熱やペストの媒介動物であるマストミス，およびペストと野兎病を媒介するプレーリードッグが輸入禁止となっていたが，その他の種類については法的な規制が存在しなかった。しかし，平成15年に感染症法が改正されたことから，野生齧歯類の輸入に対しても法的な規制が行われるようになり，わが国への野生齧歯類の輸入には輸出国側の厳格な検査が義務付けられることとなった。しかし，船舶などに紛れ込んで日本に侵入する外来性の齧歯類も存在すると考えられることから，港湾地域での齧歯類の監視活動の強化が望まれる。さらに，日本国内に存在するハンタウイルスによる人の感染状況を明らかにするために，全国規模の疫学調査を行う必要があると考えられる。

おわりに

近年，世界各国で様々なハンタウイルスが発見されつつあり，今後も新たなウイルスが次々と報告されると考えられる。前述したとおり，齧歯類とハンタウイルスは相互の結びつきが非常に強く，しかも齧歯類とハンタウイルスの系統樹が非常に近似することから，両者は地質学的な時間単位で共進化してきたと考えられている。このように齧歯

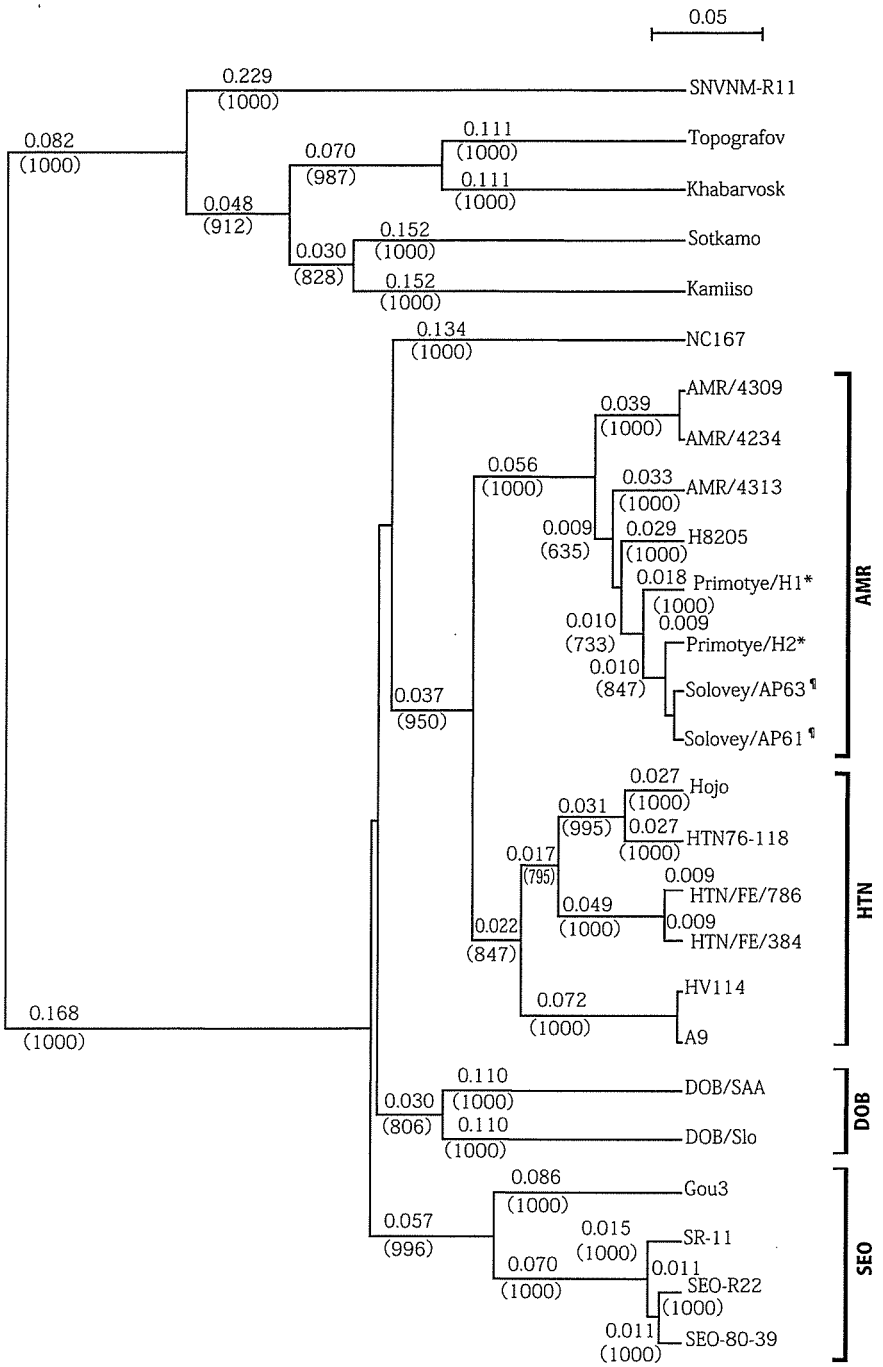

図3　ハンタウイルスのM遺伝子の系統樹解析

ウラジオストックのHFRS患者から検出されたウイルス（*）とハントウアカネズミから検出されたウイルス（¶）がアムールウイルスと共通の系統に属している。

HTN：ハンターンウイルス　AMR：アムールウイルス　DOB：ドブラバウイルス　SEO：ソウルウイルス

類とハンタウイルスは人獣共通感染症という視点からだけ　い研究課題となっている。

でなく，宿主と寄生体の相互関係という視点からも興味深

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GEOGRAPHICAL DISTRIBUTION OF HANTAVIRUSES IN THAILAND AND POTENTIAL HUMAN HEALTH SIGNIFICANCE OF *THAILAND VIRUS*

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Abstract. Phylogenetic investigations, sequence comparisons, and antigenic cross-reactivity studies confirmed the classification of *Thailand virus* (THAIV) as a distinct hantavirus species. The examination of sera from 402 rodents trapped in 19 provinces of Thailand revealed that five greater bandicoot rats (*Bandicota indica*) and one lesser bandicoot rat (*B. savilei*) from four provinces were focus reduction neutralization test (FRNT) antibody-positive for THAIV. One of 260 patients from Surin province in Thailand (initially suspected of having contracted leptospirosis, but found to be negative) showed symptoms compatible with hemorrhagic fever with renal syndrome (HFRS). The serum of this patient showed high titers of hantavirus-reactive IgM and IgG. FRNT investigations confirmed virus-neutralizing antibodies against THAIV. These observations suggest that THAIV or THAI-like viruses occur throughout Indochina and may represent an additional causative agent of HFRS.

INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) is a rodent-borne viral zoonosis caused by certain members of the viruses in the genus *Hantavirus* of the family *Bunyaviridae*.[1] The hantaviral species that have been causally associated with HFRS are *Hantaan virus* (HTNV), *Seoul virus* (SEOV), and *Dobrava/Belgrade virus* (DOBV) that are carried by the members of the rodents in the subfamily *Murinae* (Old World rats and mice), while the *Puumala virus* (PUUV) is carried by the members of the rodents in the subfamily *Arvicolinae* (voles and lemmings) of the family *Muridae*. Other hantaviral species that are not known as the causative agents of HFRS include *Tula virus* (TULV) and *Topografov virus* (TOPV) in Europe, *Khabarovsk virus* (KHAV) in far east Russia, and *Prospect Hill virus* (PHV) in the United States that are carried by rodents in the subfamily *Arvicolinae*. The *Thailand virus* (THAIV) is the only hantavirus species carried by the rodent in the subfamily *Murinae* in Thailand. *Thottapalayam virus* (TPMV) is the only hantavirus isolated from mammals in the Insectivore in India.[2]

The species of hantaviruses isolated from the rodents in the same subfamily; HTNV, SEOV, DOBV, and THAIV from rodents of subfamily *Murinae* and PUUV, TULV, TOPV, KHAV, and PHV from rodents of subfamily *Arvicolinae*, showed strong antigenic cross reactivity defined by antibody binding assays such as IFA and ELISA. Neutralization test is required to serologically distinguish among hantavirus species originated from rodents classified to the same subfamily.[3]

The THAIV strain Thai749 was originally isolated by Elwell et al. (1985) from a greater bandicoot rats (*Bandicota indica*) trapped in the vicinity of a small farm village in the western province of Kanchanaburi,[4] Thailand. Subsequent phylogenetic studies based on the nucleotide sequence of M segment of THAIV revealed that the THAIV is placed at the position most closely related to SEOV and grouped with other viruses from rodents classified to *Murinae*.[5] Thai749 strain is antigenically distinct from other hantavirus species.[3] However, only part of the nucleotide sequence information in the S segment of the THAIV is available so far.[5] For further understanding of THAIV of the relationship among other hantaviruses, nucleotide sequence information of entire S segment as well as further antigenic characterization is required.

It has been well characterized that a single rodent species or phylogenetically closely related rodent species are the principal host of a single hantavirus species.[6] The rodent fauna of Thailand includes 35 murine species in 7 genera and 1 arvicoline species, *Eothenomys melanogaster*.[7] A previous seroepizootiologic study of hantavirus infection conducted at central, northeastern, and near Bangkok areas revealed that greater bandicoot rat as a main reservoir and several species of rice field rats such as *Rattus rattus*, *exulans*, and *losea* are also natural reservoirs to a lesser extent in Thailand.[4] To extend our knowledge of the geographical distribution and natural host association of the hantaviruses in Thailand, we have continued further seroepizootiologic study, particularly by including the THAIV as antigen for serological screening.

Although the hantavirus infection spread in various species of rodents and wider areas in Thailand, epidemiologic information regarding to the human infection with hantavirus is quite limited. Suputthamongkol et al.[8] reported the first clinical case report of hantavirus infection in Thailand. However, the causative hantavirus species was not further characterized in the report. Since the clinical symptoms of leptospilosis and other febrile illness are similar to HFRS, undiagnosed HFRS cases would be existing among the patient with febrile illnesses of unknown etiology in Thailand.

In this study, we have examined antigenic and genetic prop-

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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 Dabieshan, 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′-GAIIGITGTCCACCAA-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-

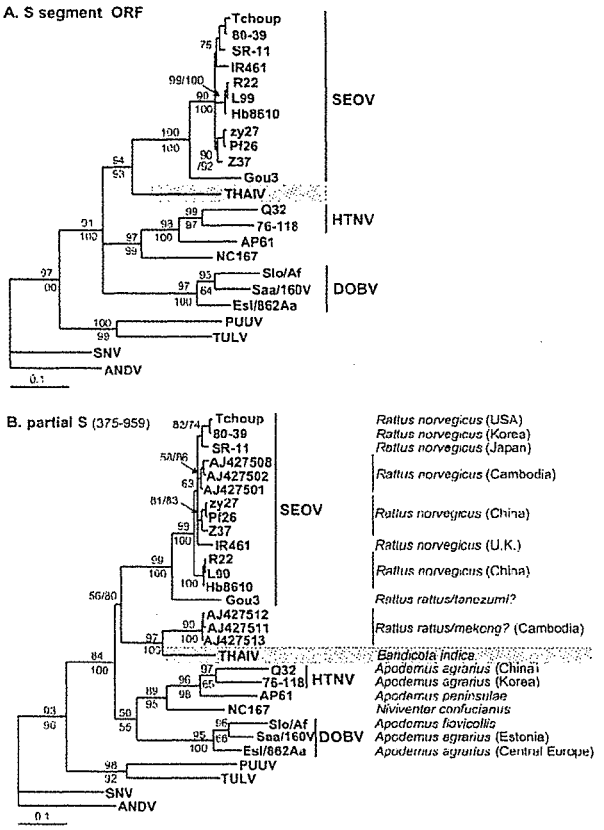

FIGURE 1. Maximum likelihood (ML) phylogenetic trees of THAIV and other Murinae-associated hantaviruses based on: (**A**) complete N protein coding nt sequences (S segment ORF); and (**B**) partial S segment nucleotide sequences of nt 375–959 (585 nts). The ML trees (Tamura-Nei evolutionary model) were 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/IR461 (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*

			HTNV			SEOV		DOBV		THAIV
Proteins	Epitope	MAbs	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.[33] All the used clones were listed as below. Cross-reactive clones for N protein: ECO2, PDO3, 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

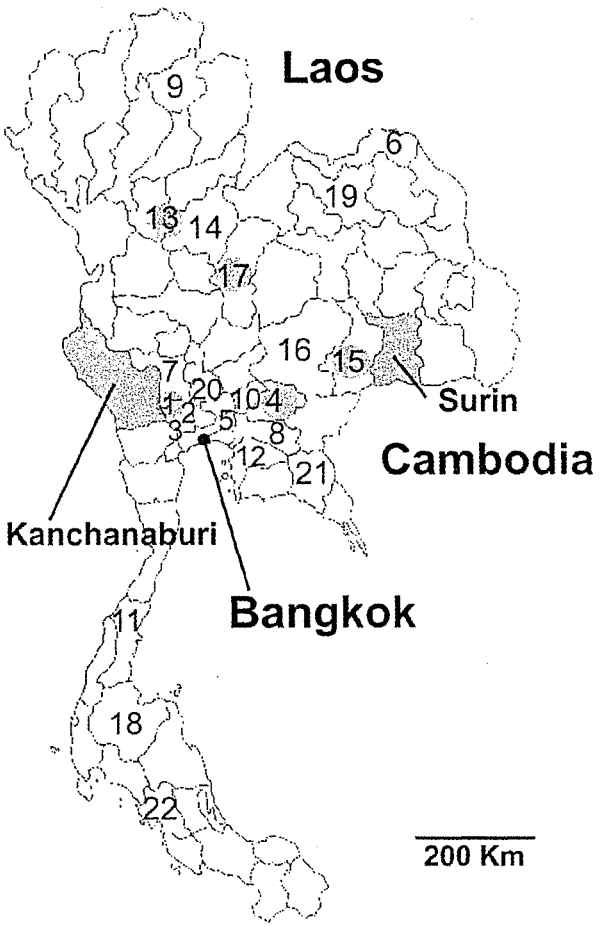

FIGURE 2. Map of Thailand showing the localization of rodent trapping sites. The numbers of the trapping sites correspond to those given in Table 1. The locations of the trapping sites where anti-hantavirus-positive rodents were captured are shown as gray circles. The geographical origin of patient #277 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.

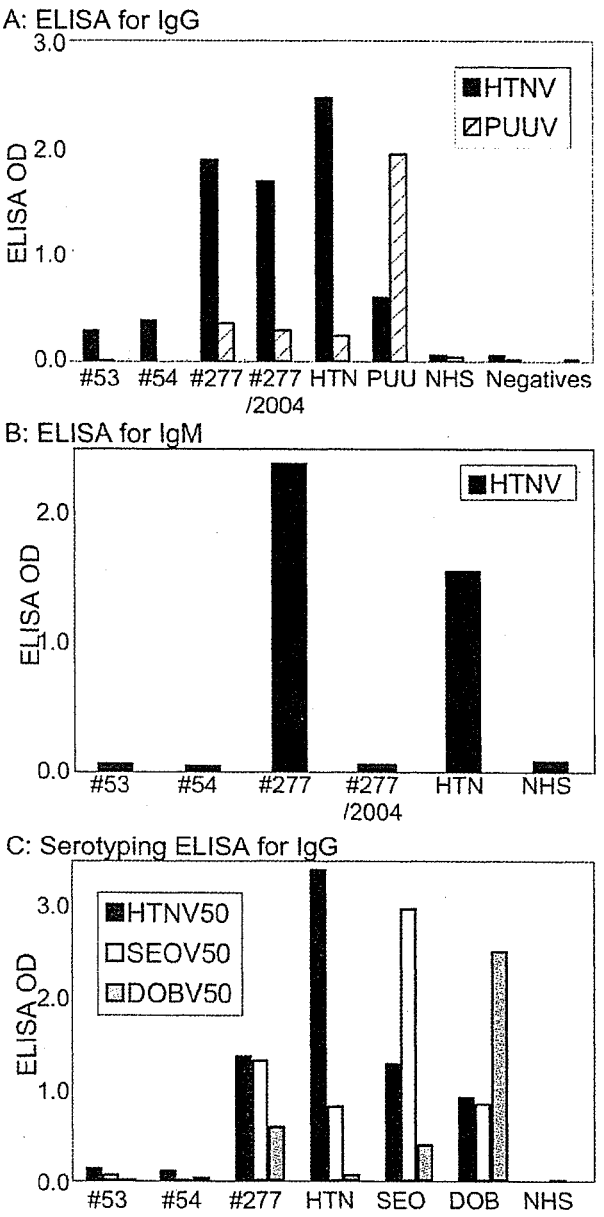

FIGURE 3. Serological screening of patient sera obtained in Thailand. (**A**) Detection of anti-hantavirus IgG in patient sera #53, #54, #277, and #277/2004 by ELISA using the recombinant N protein antigens of HTNV (closed bar) and PUUV (hatched bar). HTN and PUU are positive control sera from patients 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*.[4,37]

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.[10] 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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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'-TTCA*G AATTC* GATGA CTCAA GGGAA AATGA CTCCC GAAGA-3' and 5'-TATC*C 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.

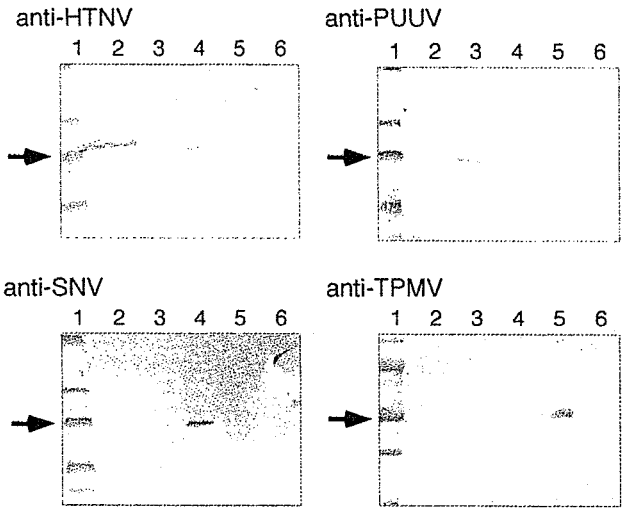

FIG. 1. Western blot analysis of hantavirus antigens using polyclonal rabbit immune sera. We tested the cross-reactivity of each hantavirus, including TPMV, using sera from rabbits immunized with rN antigens expressed in *E. coli*. For HTNV, PUUV, and TPMV antigens, viruses were inoculated on Vero E6 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.

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.

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:1 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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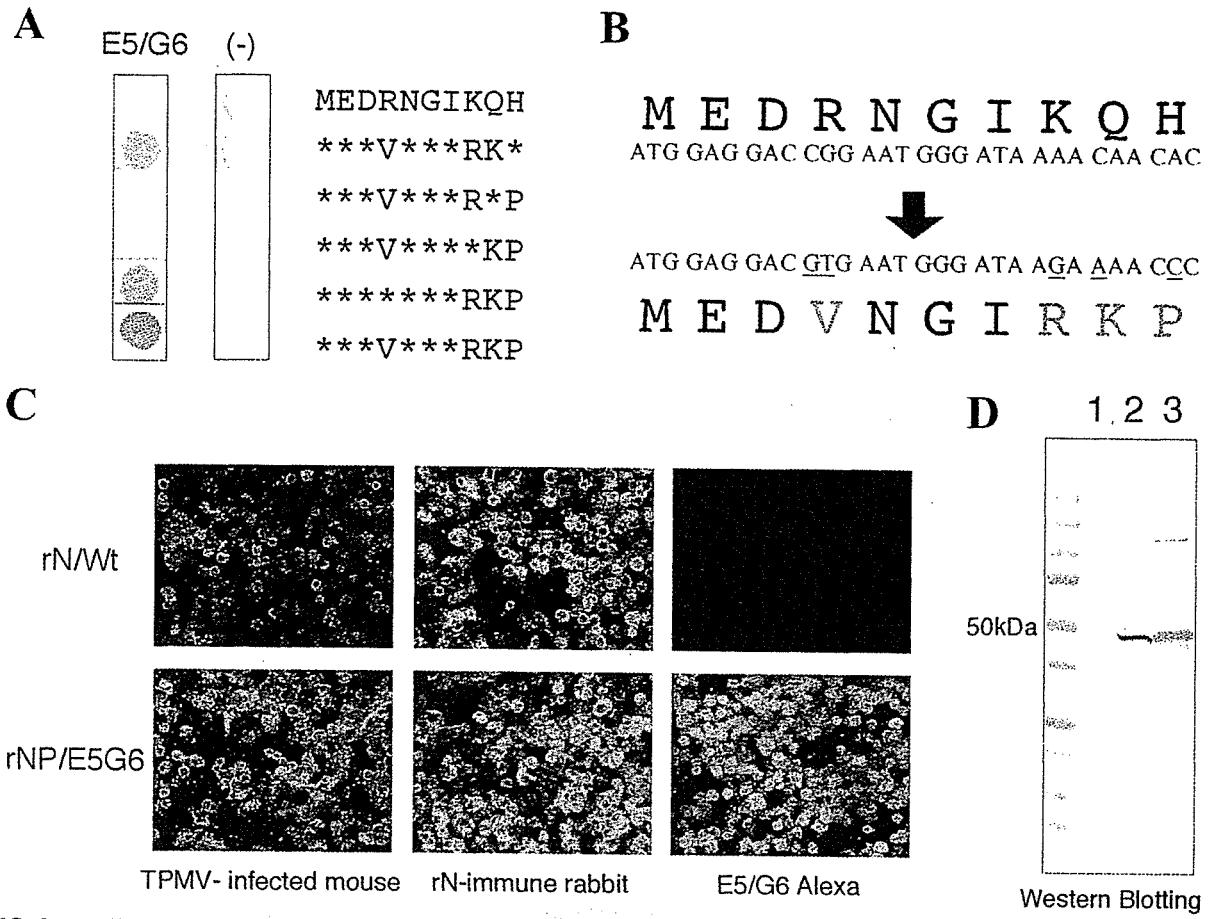

FIG. 2. A. E5/G6 epitope analysis of TPMV N. Using a variety of synthesized 10-mer peptides, we confirmed E5/G6 reactivity against TPMV sequence. Further, we determined which amino acid changes in this region were essential for E5/G6 binding. The peptide changes at positions 178 (R→V), 182 (K→R), 183 (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, F2 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.

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

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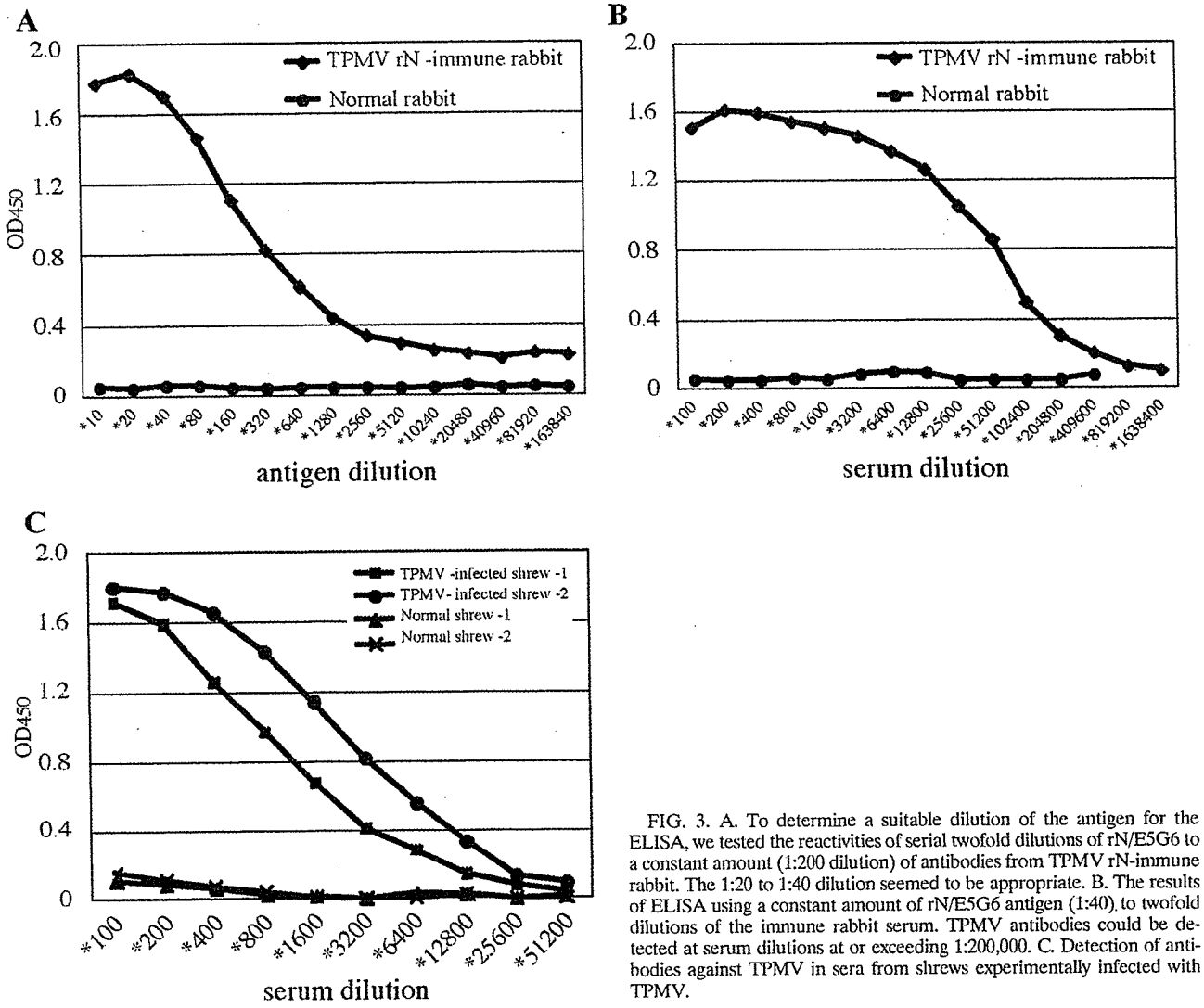

FIG. 3. A. To determine a suitable dilution of the antigen for the ELISA, we tested the reactivities of serial twofold dilutions of rN/E5G6 to a constant amount (1:200 dilution) of antibodies from TPMV rN-immune rabbit. The 1:20 to 1:40 dilution seemed to be appropriate. B. The results of ELISA using a constant amount of rN/E5G6 antigen (1:40) to twofold dilutions of the immune rabbit serum. TPMV antibodies could be detected at serum dilutions at or exceeding 1:200,000. C. Detection of antibodies against TPMV in sera from shrews experimentally infected with TPMV.

E6 cell antigen. So, we succeeded in producing TPMV rN with the E5/G6 epitope, which has the antigenicity of TPMV as well as reactivity with MAb E5/G6.

Developing the E5/G6 capture ELISA system for TPMV. Using the TPMV rN/E5G6 antigen, we developed an IgG antibody-detecting capture ELISA system, according to methods described previously (1, 7, 8, 18). Figure 3A shows the results of an ELISA with twofold dilutions of TPMV rN/E5G6 to a constant dilution of rN-immune rabbit sera (1:200 dilution), and Fig. 3B shows the results of an ELISA with a constant amount of TPMV rN/E5G6 antigen (1:40 dilution) to serial twofold dilutions of rN-immune rabbit sera. The ELISA assay system using the TPMV rN/E5G6 antigen detected anti-TPMV antibodies with high sensitivity. Results with sera from shrews experimentally infected with TPMV also supported the sensitivity of this assay system (Fig. 3C).

In addition, we compared the antigenic cross-reactivities of TPMV and other hantaviruses using this ELISA system (Table 2). In the reactions with each homologous combination, the optical density value was remarkably high. Although the heterologous combinations showed a variety of reactivities, according to the antigenic similarity between viruses, TPMV antigen did not cross-react with other antihantavirus antibodies. This result indicated that rN/E5G6 is a useful tool for the specific detection of anti-TPMV antibodies.

Serological survey of TPMV infection among febrile patients in Thailand. Employing the newly developed capture ELISA

TABLE 2. Cross-reactivities in capture ELISA among TPMV and representative disease-causing hantaviruses

Source of antigen	Cross-reactivity of immune rabbit serum to:			
	HTNV	PUUV	SNV	TPMV
HTNV	0.781	0.453	0.037	0.015
PUUV	0.671	1.487	0.669	0.000
SNV	0.614	1.362	1.672	0.036
TPMV	0.011	0.007	0.002	1.578

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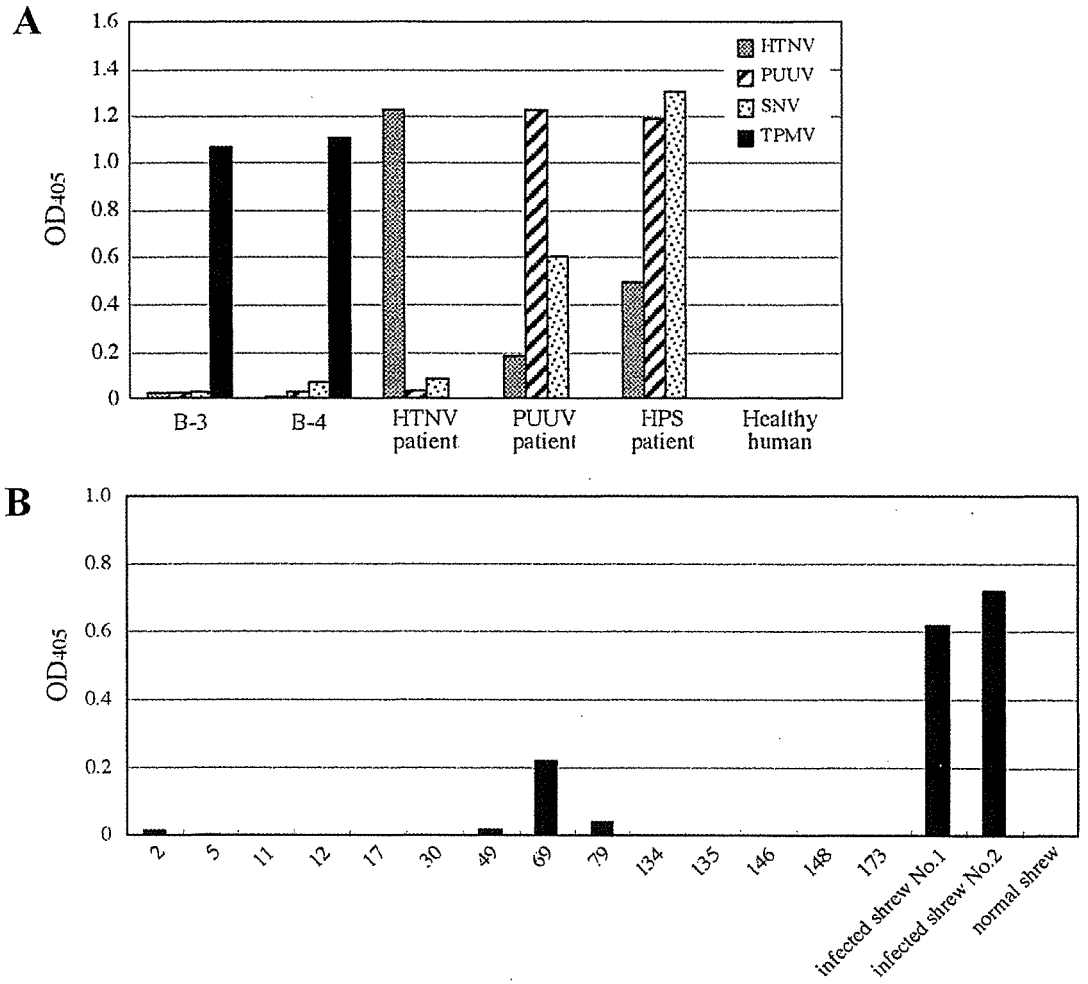

FIG. 4. Serological surveys for TPMV infection in Southeast Asia. We examined 478 sera from patients with fever in Thailand and found two sera (B-3 and B-4 from the same patient at different phases of illness) with anti-TPMV IgG antibodies. Results of the ELISA are shown in panel A. Sera B-3 and B-4 reacted with TPMV antigen. HTNV, PUUV, and HPS patient immune sera served as positive controls. In addition, we tested sera from 14 wild shrews (*Suncus murinus*) captured in Indonesia in 2005 (B). Serum no. 69 was positive for anti-TPMV IgG antibodies, while sera no. 2, 49, and 79 were weakly positive.

system, we tested 478 serum samples from patients with fever in Thailand who were serologically negative for leptospirosis and dengue fever. Each serum was tested with whole HTNV, PUUV, SNV, and TPMV rN for serotyping (Fig. 4A). Serum samples no. B-3 and no. B-4, which were from the same patient during different phases of illness, were positive for anti-TPMV IgG antibodies. Sera from seven other cases were weakly positive to HTNV (data not shown). Sera no. B-3 and no. B-4 also reacted with TPMV-infected Vero E6 cells by the IFA test and Western blot analysis (Fig. 5A and B) and by FRNT using native TPMV (Table 3). However, virus-specific IgM was not detected (data not shown). Therefore, this patient may have been infected with TPMV previously, although it is not clear if he had shown symptoms.

This anti-TPMV-antibody-positive patient was a 58-year-old Laotian male who fell ill in Laos and came to a hospital in Nongkhai Province, along the border of Thailand and Laos, in April 2005. He presented with high fever, chills, headache, cough, sore throat, vomiting, diarrhea, abdominal pain, and exhaustion. The patient recovered fully after being hospitalized for several weeks. However, these symptoms were not necessarily related to TPMV infection directly, because he lacked IgM against TPMV. Unfortunately, no information is available about his occupation or his exposure to shrews or wildlife.

Serological survey of TPMV infection in wild shrews captured in Indonesia. Of sera collected from 14 shrews captured in Indonesia in 2005, one (no. 69) was positive for anti-TPMV IgG antibodies by ELISA. Sera from three other shrews (no. 2, 49, and 79) were weakly positive by ELISA (Fig. 4B). By contrast, in the IFA test using TPMV-infected Vero E6 cells as antigen, sera no. 49 and 69 were positive (Fig. 5A), whereas sera no. 2 and 79 were negative. Sera no. 49 and 69 were also positive by Western blotting analysis using TPMV-infected Vero E6 cell antigens (Fig. 5B). Only no. 49 was positive by FRNT (Table 3).

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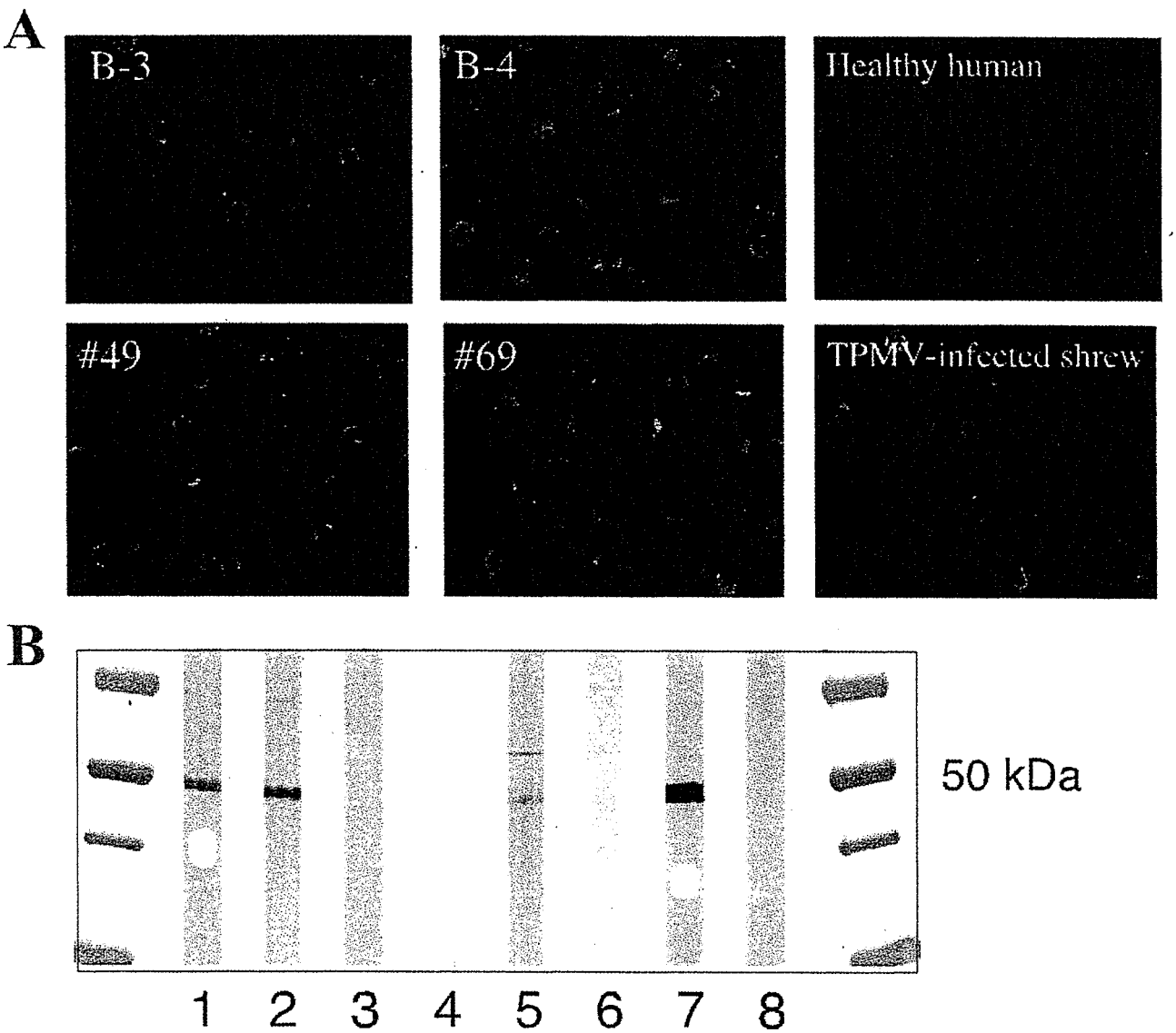

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AQ: E FIG. 5. A. Results of IFA test using TPMV-infected Vero E6 cell antigens. As a positive shrew serum control, serum from a shrew experimentally infected with TPMV was used. Sera no. 49 and 69 were positive against TPMV antigen. But sera no. 2 and 79 were negative by the IFA test (data not shown). B. Western blot analysis of TPMV-positive sera with TPMV antigen. The human positive sera B-3 and B-4 in ELISAs also reacted with TPMV-infected Vero E6 antigen by Western blot analysis. On the other hand, for shrews, only no. 49 and 69 showed a band at 50 kDa with TPMV antigen, and sera no. 2 and 79 did not. Lanes 1 (B-3) and 2 (B-4) are human positive samples in ELISA; lane 3 (no. 2), lane 4 (no. 49), lane 5 (no. 69), and lane 6 (no. 79) are shrew positive samples in ELISA; lane 7 (serum from a shrew experimentally infected with TPMV) is a positive control. Lane 6 (normal shrew serum) is a negative control.

TABLE 3. FRNT with native TPMV in human and shrew sera[a]

Serum no.	FRNT titer
Human	
B-3	40
B-4	80
(−)	<40
Shrew	
49	80
69	<40
(+)	320
(−)	<40

[a] Human (−) is a serum from a healthy individual as a negative control. Shrew (+) is a serum from a shrew experimentally infected with TPMV as a positive control, and (−) is a serum from a normal uninfected shrew.

DISCUSSION

Long unclassified, TPMV is now known to be a member of the genus *Hantavirus*. Surprisingly little is known about TPMV, however, despite the fact that its isolation predates that of Hantaan virus. For example, until very recently sequences of the full-length S-, M- and L-genomic segments of TPMV were not known. Also, although TPMV was isolated from tissues of a musk shrew, the identity of its natural reservoir host has remained shrouded in some uncertainty, with some believing that TPMV must represent spillover from a rodent host. The dearth of information about TPMV can largely be attributed to