INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) is a rodent-borne viral zoonosis caused by certain members of the genus Hantavirus of the family Bunyaviridae. 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 Murininae (Old World rats and mice), while the Psammale virus (PUUV) is carried by the members of the rodents in the subfamily Arvicolinea (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 Arvicolinea. The Thailand virus (THAIV) is the only hantavirus species carried by the rodent in the subfamily Murininae in Thailand. Thottapalayam virus (TPMV) is the only hantavirus isolated from marmots in the Insectivore in India. The species of hantaviruses isolated from the rodents in the same subfamily; HTNV, SEOV, DOBV, and THAIV from rodents of subfamily Murininae and PUUV, TULV, TOPV, KHAV, and PHV from rodents of subfamily Arvicolinea, 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.

The THAIV strain Thai749 was originally isolated by Elwell et al. (1985) from a greater bandicoot rat (Bandicota indica) trapped in the vicinity of a small farm village in the western province of Kanchanaburi, 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 Murininae. Thai749 strain is antigenically distinct from other hantavirus species. However, only part of the nucleotide sequence information in the S segment of the THAIV is available so far. 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. The rodent fauna of Thailand includes 35 murine species in 7 genera and 1 arvicoline species, Eothenomys melanogaster. 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 lusea are also natural reservoirs to a lesser extent in Thailand. 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. Suputhammongkol et al. 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 leptospirosis 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-
Hantavirus Infection in Indochina

Rattus

Since Hantaviruses possess a negative-sense RNA genome

In addition, bootstrap

/H11032 – species in Thailand.

/H11032 – Hantaan virus

Rattus rattus

The nucleotide se-

15

18

11

1

12

13

14

15

16

17

18

DBSV)-NC167 isolated from 

E. coli

20

11

11

19

19

11

11

/HTNV strain

995

11

11

vector using the Original TA

were used for antigenic com-

Briefly, acetone-fixed

16

17

18

(HTNV) strain 76-118

11

11

Clones that produce

12,13

captured in a mountainous region near Dabi-

sion. The PCR products derived from the S genome segment

were cloned into an

Cloning Kit (Invitrogen, Carlsbad, CA). Two clones of each

were cloned into an

tion. The PCR products derived from the S genome segment

with the same primers that were used for the PCR amplifica-

derived from the partial M segment was purified using a PCR

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,19 Saaremaa-DOE,16 SEOV strain Gou3,11 and HTNV strain Da Bie Shan virus (DBSV)-NC167 isolated from Niviventer confucianus captured in a mountainous region near Dabi-

shan, Anhui Province, China,11 were used for antigenic compar-

isons.

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.9 The nucleotide sequence of the M segment that corresponds to nucleotides (nt) 2000–2300, the primer pair THLM1910F, (5′-AAGACAA-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′-TTTTCAAGTGACACTT-3′) and CS6 (5′-TTTTCAAGTGACACTT-3′), as well as GS4 (5′-GAIGITGTCCACCAA-CATG-3′) and CS8 (5′-GAIGITGTCCACCAA-CATG-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 se-

quenced 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 CLUSTALW15 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 phyloge-

netic 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 package16,17 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 soft-

ware 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 Tree-

View (Glasgow, UK) v.1.6.6 (http://taxonomy.zoology.gla.ac.

uk/rod/treeview.html). The accession numbers of the se-

quences 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 se-

quence 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 immuno-

globulin 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 strain 76-118, SEOV strain SR-11, and THAIV strain Thai749, were deter-

mined by FRNT, as described earlier.20 For this purpose, we selected seropositive sera from human and rodent sera. Hu-

man 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 inves-

tigation. However a Rattus rattus serum from Petchaburi was not used for FRNT assay because its amount was not suffi-
In total, 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. 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 recombinant bovine 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 H₂SO₄. 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. 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 characteristic fluorescence was regarded as positive. In IFA test, the sera were examined at 1:40 dilution with HTNV strain 76-118-infected Vero E6 cell smear 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 76-118 as described previously. As positive controls, 3 serum samples from Wistar rats that were experimentally in-
HANTAVIRUS INFECTION IN INDOCHINA

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

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Epitope</th>
<th>MAbs</th>
<th>HTNV</th>
<th>AMRV</th>
<th>DBSV</th>
<th>DOBV</th>
<th>DOBV</th>
<th>THAIV</th>
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<tr>
<td></td>
<td></td>
<td>76118</td>
<td>H5</td>
<td></td>
<td>NPC1</td>
<td>Slovenia</td>
<td>Saaremaa</td>
<td>Thai749</td>
</tr>
<tr>
<td>N</td>
<td>Cross-reactive</td>
<td>ECO2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Genus-common</td>
<td>ES6G</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>HTNV-specific</td>
<td>BDO1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>SEOV-specific</td>
<td>DC03</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>G1/G2</td>
<td>G1a</td>
<td>6D4</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>G1b</td>
<td>3D5</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>G2a</td>
<td>HCO2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>G2b</td>
<td>EBO6</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>G2c</td>
<td>11E10</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>G2d</td>
<td>3D7</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td></td>
<td>G2e</td>
<td>20D3</td>
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<td>++</td>
</tr>
<tr>
<td></td>
<td>G2f1</td>
<td>1G8</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>G2f2</td>
<td>7G6</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

* Binding profiles of clones data not shown in this table were basically same results as representative clones and previous reports. All the used clones were listed as below. Cross-reactive clones for N protein: ECO2, DFO3, KA06, ECO1, GBO4, C16D11, and F23A1; Genus-common epitope binding clone: ESG6; HTNV-specific clones for N protein: BDO1, C24B4, and G5; SEOV-specific clone for N protein: DC03.

Clones for glycoprotein epitope G1a: 6D4, 8B6, and 10F11; G1b: 3D5, 25D, and 16D2; G2a: HCO2 and 16F6; G2b: EBO6; G2c: 11E10; G2d: 3D7; G2e: 20D3, 17G6 and 5B7; G2f1: 1G8, SE010, 1C6, 2G10-2, and 3B6; G2f2: 7G6, 23G10-1, and 18F5. Designations: −, < 10; +, 10^-118), SEOV (strain SR-11), and DOBV (strain Saaremaa) and truncated 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. 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), were confirmed to contain no antibodies against hantaviruses, were used.

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). Partial S 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 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).

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. 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.

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. 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

affected with SEOV strain SR-11 were used. As negative controls, sera from 5 wild-trapped, non-infected rats from Japan were used.

Human sera and methods for antibody detection. Screening for anti-hantavirus IgG and serotyping were performed by ELISA tests, as previously described, 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, borovirus strain 76-118, SEOV (strain SR-11), and DOBV (strain Saaremaa) 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. 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), were confirmed to contain no antibodies against hantaviruses, were used.

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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 Bandicota indica, one from Bandicota savilei, and one from Rattus rattus. The 5 provinces in which the seropositive rodents were located (i.e., Nakhon Pathom, Prachin Buri, Suphan Buri, Chanthaburi, 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 other positive sera, one from Bandicota indica and one from Rattus 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,
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.

### Table 3

<table>
<thead>
<tr>
<th>Serum specimen/antiserum</th>
<th>Reciprocal end-point titer† against HTNV</th>
<th>SEOV</th>
<th>THAIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bi56 (B. indica)</td>
<td>&lt;40</td>
<td>&lt;40</td>
<td>≥1280</td>
</tr>
<tr>
<td>Bi57 (B. indica)</td>
<td>&lt;40</td>
<td>&lt;40</td>
<td>≥1280</td>
</tr>
<tr>
<td>Bi58 (B. indica)</td>
<td>&lt;40</td>
<td>&lt;40</td>
<td>80</td>
</tr>
<tr>
<td>Bi55 (B. savilei)</td>
<td>&lt;40</td>
<td>&lt;40</td>
<td>160</td>
</tr>
<tr>
<td>Anti-HTNV/mice</td>
<td>≥1280</td>
<td>&lt;40</td>
<td>&lt;40</td>
</tr>
<tr>
<td>Anti-SEOV/rat</td>
<td>&lt;40</td>
<td>≥1280</td>
<td>80</td>
</tr>
<tr>
<td>Negative sample of B. indica</td>
<td>&lt;40</td>
<td>&lt;40</td>
<td>&lt;40</td>
</tr>
<tr>
<td>#277</td>
<td>640</td>
<td>&lt;40</td>
<td>160</td>
</tr>
<tr>
<td>Anti-SEOV</td>
<td>80</td>
<td>640</td>
<td>160</td>
</tr>
<tr>
<td>NHS‡</td>
<td>&lt;40</td>
<td>&lt;40</td>
<td>&lt;40</td>
</tr>
</tbody>
</table>

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

![Figure 3](image-url)
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. 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 hamagglutination 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. The definition of THAIV as a distinct species was based on its association to a unique rodent species (i.e., B. indica). Recently, THAIV genome was amplified by RT-PCR from B. indica captured in central Thailand (personal communication from Alexander Plyphun). 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. Therefore, this report provides additional support for defining THAIV as a distinct species among the hantaviruses.

Schmaljohn et al. 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. 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. 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 Polynesians rats (Rattus exulans). Therefore, search for THAIV or THAI-like viruses should be extended to southern provinces as well as neighboring Cambodia. 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 epidemiological 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 THAI-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. Ewell et al. 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. 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. 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.

Although a recent publication attributed a first clinical case to hantavirus infection in Thailand, 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. In most hantiviral disease cases, both IgM and IgG to hantavirus are positive at the onset of clinical disease. 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, 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. and our study indicate that human disease caused by hantiviruses 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. 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. Molecular, epidemiologic, and serological studies on hantiviruses in rodents and humans have also been reported from Cambodia and Indonesia. Taken together, this indicates a wide distribution and potentially high diversity of hantiviruses in Southeast Asia calling for further studies on human hantavirus infections, its rodent reservoirs, and possible transmission routes.

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