**FULL PAPER** *Public Health*

# Studies on Hantavirus Infection in Small Mammals Captured in Southern and Central Highland Area of Vietnam

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(Received 20 December 2011/Accepted 28 April 2012/Published online in J-STAGE 22 May 2012)

ABSTRACT. To investigate the distribution of hantaviruses among animals in Southern and Central Highland area of Vietnam, a total of 1311 serum samples were obtained from rats and Asian house shrews (*Suncus murinus*) captured at 11 locations between 2006 and 2009. A total of 1066 serum samples from rats were examined for IgG antibodies against Hantaan virus, and there were 30 antibody-positive serum samples from rats that had been captured mainly in a port area and urban area in Ho Chi Minh City (HCMC) (2.8%). All of the antibody-positive rats were *Rattus norvegicus*, and they had Seoul virus (SEOV) genome in their lungs. SEOV sequences detected from rats captured in Southern Vietnam belonged to the same lineage as those from rats captured at Haiphong Port and a market area in Hanoi City. SEOV strain CSG5 was isolated from a rat captured at Saigon Harbor. Strain CSG5 showed a cross-neutralization pattern almost the same as that of a representative strain of SEOV. A total of 245 Asian house shrews were captured in the Central Highland area and near HCMC. Sera were examined for IgG antibodies against Thottapalayam virus (TPMV), and 32 (13.1%) of the antibody-positive shrews were mainly from the Central Highland area and showed a neutralizing antibody against TPMV. These results indicated that SEOV is distributed among *R. norvegicus* inhabiting harbor and urban areas of Southern Vietnam and that TPMV or an antigenically related virus is distributed among Asian house shrews in Central Highland area.
KEY WORDS: epidemiology, hantavirus, rat, shrew, zoonosis.

doi: 10.1292/jvms.11-0567; *J. Vet. Med. Sci.* 74(9): 1155–1162, 2012

Hantaviruses belong to the *Hantavirus* genus in the *Bunyaviridae* family. Hantaviruses are enveloped RNA viruses that contain three-segmented negative-sense RNAs, designated S, M and L on the basis of their molecular sizes in their virion. The S, M and L RNA segments encode nucleocapsid protein (N), enveloped glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase (L) protein, respectively [20].

Various hantaviruses have been isolated from rodents, which are persistently infected with these viruses. Usually, each hantavirus appears to have a single predominant species of rodent as a natural reservoir. Phylogenetic analyses of nucleotide sequences of the viral genome and rodent mitochondrial DNA have demonstrated a close correlation, suggesting co-evolution of the virus and host [10, 15].

Hantaviruses include causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Viruses are transmitted to humans from reservoir rodents via excreta. Causative agents of HFRS including Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava/Belgrade virus, Thailand virus (THAIV) and Puumala virus (PUUV) are distributed throughout Europe, Russia, and East Asia. On the other hand, all of the causative agents of HPS are confined to New World rodents belonging to the subfamilies Neotominae and Sigmodontinae [19].

Although HFRS cases have been reported throughout the Eurasia continent, more than 90% of the cases have been reported in Far Eastern countries including China, Russia and Korea [9]. In other East Asian countries, such as Thailand, Indonesia, Vietnam, Sri Lanka and India, prevalence of SEOV and THAIV infection has been shown among rodents and humans [2, 3, 6, 13, 14, 16, 17, 22].

Thottapalayam virus (TPMV), one of the members of the genus *Hantavirus*, was isolated from an Asian house shrew (*Suncus murinus*) captured in 1964 in southern India [1]. Since most of the hantaviruses have been isolated from rodents, TPMV has been considered as an exception that is harbored by animals classified to a different order, Soricomorpha, after transmission from the rodent host to shrew by a spillover event. However, hantaviruses that are antigenically and genetically distinct from rodent-borne hantaviruses have recently been isolated from at least 11 different species of shrew or mole worldwide, which have been classified into the order Soricomorpha [7]. However, the significance of Soricomorpha-derived hantaviruses in human infection remains unclear.

In Vietnam, SEOV infection among port workers and rats in Hai Phong Port and among residents and rats in Hanoi City including surrounding urban areas has been reported

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[15]. Furthermore, we have reported a HFRS case of SEOV infection in Ho Chi Min City (HCMC) [2]. Interestingly, seropositive persons in rural areas were suspected to have been infected with a hantavirus that was antigenically different from previously reported hantaviruses such as SEOV, HTNV and THAIV [22]. That report indicated that a variety of novel hantaviruses might exist in Vietnam. However, information regarding the hantavirus and reservoir relationships is still limited.

In the present study, we screened for antibodies against Murinae-rodents-associated and Soricidae-associated hantaviruses in rodents and shrews captured in Southern Vietnam and Central Highland area.

## MATERIALS AND METHODS

*Viral strains and cells*: Strains 76-118, SR-11, Thai749 and VRC-66412 were used as representative HTNV, SEOV, THAIV and TPMV species, respectively. SEOV, THAIV, and TPMVs were propagated in Vero cells (clone E6; ATCC C1008) prior to use in a focus reduction neutralization test (FRNT) described below.

*Animal sera*: A total of 1,311 serum specimens were obtained from 4 different *Rattus* species (*R. norvegicus, R. exulans, R. argentiventer* and an unidentified *Rattus* species) captured at 6 locations and from Asian house shrews (*Suncus murinus*) captured at 4 locations in the period from 2006 to 2009 (Table 1 and Fig. 1). Species of animals were determined first by morphological characteristics, and then mitochondrial DNA sequencing was carried out in representative animals as previously described [18].

*Antibody detection*: All of the sera were screened by ELISA. Then, ELISA-positive and -intermediate specimens were confirmed by IFA and Western blot. Finally, sera showing positive reactions in 3 tests were regarded as antibody-positive.

*ELISA*: Sera were screened by indirect IgG ELISA using essentially the same method as that previously described. Briefly, *Escherichia coli*-expressed His- and NUS-tagged partial N protein (103 amino acids from the N-terminus) from HTNV strain 76-118 or full-length N protein from TPMV expressed using a pET43.1 vector system (Novagen) was used as previously described [13]. Since the N-termini of N proteins of HTNV, SEOV and THAIV possess a common antigenic region, N-terminus of N protein of HTNV was used as a representative antigen for screening antibodies to either virus. The sera were then diluted 1:200 and screened by ELISA. The presence of bound antibodies was detected using a horseradish peroxidase-conjugated goat anti-rat IgG antibody (Zymed Laboratories Inc. South San Francisco, CA, U.S.A.) or Protein A (Prozyme, Heyward, CA, U.S.A.). OD values higher than 0.2 with horseradish peroxidase-conjugated goat anti-rat IgG antibody and higher than 0.1 with horseradish peroxidase-conjugated Protein A were used as cut off values between positive and negative. To detect hantavirus-specific IgM antibody in rat sera, IgM ELISA was performed as previously described [24]. As negative controls, sera from five wild-trapped, uninfected rats from Japan were used [21].

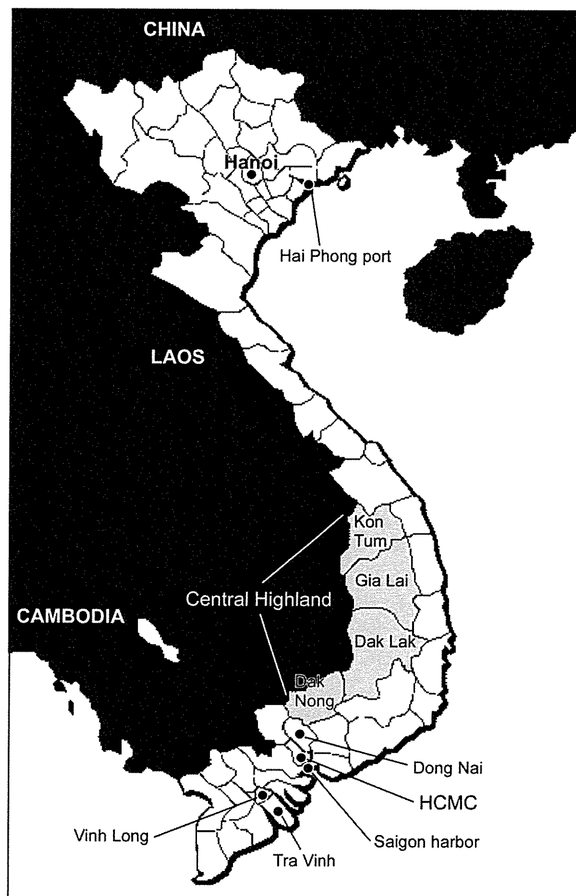


Fig. 1. Map of Vietnam showing the rodent and shrew trapping sites. The locations of the Central Highland area are shown in gray. District 12 and Saigon Harbor shown in Table 1 are in Ho Chi Minh City (HCMC).

*Western blotting.* The antibody-positive sera were then subjected to Western blotting using recombinant N proteins from HTNV and TPMV expressed by a baculovirus vector, as described previously [13, 27]. Sera were examined at 1:100 dilutions.

*Indirect immunofluorescent antibody (IFA) assay.* The IFA assay was performed as described previously [26]. Briefly, acetone-fixed smears of Vero E6 cells infected with SEOV strain SR-11 or TPMV strain VRC-66412 were used as antigens. To detect antibodies bound to the antigen, FITC-conjugated anti-rat IgG (Zymed Co, Ltd.), anti-human IgG (Zymed), and Protein A (Prozyme) were used, as described previously [8]. Serum samples showing characteristic fluorescence in infected Vero E6 cells at 1:100 dilution, but that were negative with uninfected Vero E6 cells, were regarded as positive.

*Focus reduction neutralization test (FRNT).* The FRNT was carried out by the previously described method [9]. SEOV, THAIV and TPMV were used. Briefly, 100 $\mu l$ of serial twofold dilutions of serum was mixed with an equal

Table 1. Seropositivities of animals captured from Central and Southern Vietnam

| Location | Province | Year | Rodents | | | | | | | | | | | Shrew | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *R. norvegicus* | | *R. exulance* | | *R. argentiventer* | | *R. spp** | | Subtotal | | | *S. murinus* | | |
| | | | Positive | Tested | Positive | Tested | Positive | Tested | Positive | Tested | Positive | Tested | Positive (%) | Positive | Tested | Positive (%) |
| Southeast | Dongnai | 2006 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.0% | 2 | 16 | 12.5% |
| | | 2007 | 2 | 38 | 0 | 29 | 0 | 0 | 0 | 0 | 2 | 67 | 3.0% | 0 | 12 | 0.0% |
| | | 2008 | 0 | 8 | 0 | 83 | 0 | 0 | 0 | 3 | 0 | 94 | 0.0% | 0 | 3 | 0.0% |
| | Saigon Harbor | 2007 | 10 | 57 | 0 | 4 | 0 | 10 | 0 | 0 | 10 | 71 | 14.1% | 0 | 0 | 0.0% |
| | | 2008 | 13 | 54 | 0 | 5 | 0 | 10 | 0 | 0 | 13 | 69 | 18.8% | 0 | 1 | 0.0% |
| | District 12-HCMC | 2008 | 5 | 16 | 0 | 8 | 0 | 3 | 0 | 5 | 5 | 32 | 15.6% | 0 | 0 | 0.0% |
| Central Highland | Daklak | 2007 | 0 | 1 | 0 | 54 | 0 | 0 | 0 | 0 | 0 | 55 | 0.0% | 0 | 9 | 0.00% |
| | | 2008 | 0 | 0 | 0 | 50 | 0 | 0 | 0 | 10 | 0 | 60 | 0.0% | 1 | 10 | 10.00% |
| | | 2009 | 0 | 0 | 0 | 28 | 0 | 0 | 0 | 1 | 0 | 29 | 0.0% | 0 | 9 | 0.00% |
| | Gialai | 2007 | 0 | 1 | 0 | 268 | 0 | 13 | 0 | 8 | 0 | 290 | 0.0% | 5 | 72 | 6.90% |
| | | 2008 | 0 | 0 | 0 | 39 | 0 | 6 | 0 | 0 | 0 | 45 | 0.0% | 1 | 20 | 5.00% |
| | | 2009 | 0 | 0 | 0 | 38 | 0 | 0 | 0 | 6 | 0 | 44 | 0.0% | 14 | 38 | 36.80% |
| | Kontum | 2007 | 0 | 0 | 0 | 19 | 0 | 1 | 0 | 2 | 0 | 22 | 0.0% | 9 | 36 | 25.00% |
| | | 2009 | 0 | 0 | 0 | 20 | 0 | 0 | 0 | 5 | 0 | 25 | 0.0% | 0 | 10 | 0.00% |
| | Daknong | 2009 | 0 | 0 | 0 | 71 | 0 | 0 | 0 | 2 | 0 | 73 | 0.0% | 0 | 3 | 0.00% |
| Mekong Liver Delta | Travinh | 2007 | 0 | 4 | 0 | 38 | 0 | 0 | 0 | 6 | 0 | 48 | 0.0% | 0 | 6 | 0.0% |
| | Vinhlong | 2008 | 0 | 0 | 0 | 17 | 0 | 17 | 0 | 8 | 0 | 42 | 0.0% | 0 | 0 | 0.0% |
| Total | | | 30 | 179 | 0 | 771 | 0 | 60 | 0 | 56 | 30 | 1066 | 2.8% | 32 | 245 | 13.1% |

*R. spp; R. kortaensis (R. andamanensis), R. rattus, R. tanezumi, R. rattus diardii, and Bandicorta indica. Because of lacking of tissue specimen, moclecular biological anlaysis was not carried out.

volume of virus suspension containing 200 focus-forming units (FFU) of virus at 37°C for 1 hr. Fifty $\mu l$ 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 hr 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 had been immunized with a truncated N protein (amino acids 1 to 244) of HTNV, followed by the addition of horseradish peroxidase-labeled goat antibodies against rabbit IgG and substrate. The FRNT titer was expressed as the reciprocal of the highest serum dilution that resulted in more than 80% reduction in the number of infected cell foci. As positive controls, three serum samples from WKAH/slc rats (SLC, Hamamatsu, Japan) that had been experimentally infected with SEOV strain SR-11 were used [24]. For the shrew sera, serum samples from shrew experimentally infected with TPMV and uninfected shrew serum samples were used as positive and negative controls, respectively [13].

*PCR, nucleotide sequencing, and phylogenetic analysis*: Total RNA from rat lung tissue was isolated and cDNA were synthesized as described previously [23]. Nucleotides (nt) 2000–3101 of the M genome segment, which encodes the Gc region of envelope glycoprotein, were amplified using the primers SEOMF1936 (5′-GTGGACTCTTCTTCTCATTATT-3′) and M12 (5′-AACCACTATGGCCACCTTTC-3′). The entire S segment was amplified with primer pairs CS1 (TAGTAGTAGACTCCCTAAAGAGCTAC) and CS8 (TAGTAGTAGGCTCCCTAAAAAGACAA) as described previously [23]. All products of the expected size were purified using a PCR purification kit (Qiagen, Hilden, Germany) and sequenced using the original PCR primers. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit (ver. 3.1; Perkin Elmer) with a model 3100 DNA Sequencing System (Perkin Elmer) by Hokkaido System Science Co., Ltd., Sapporo, Japan. The sequences were aligned using CLUSTALW and Genetyx Mac (ver. 13.0.6) with default parameters (gap insert penalty, −12; gap extend penalty, −4). In subsequent phylogenetic analyses, neighbor-joining (NJ) phylogenetic and bootstrap analyses were calculated by Genetyx Mac (ver. 13.0.6) and/or Phylip (ver. 3.65).

*Virus isolation*: Ten percent (v/v) of the homogenate of lung tissue with PBS was inoculated into 6-week-old female FOX CHASE SCID C.B-17/lcr-scid/scidJcl mice (CLEA Japan Inc., Tokyo, Japan). All animals were handled according to the Laboratory Animal Control Guidelines of the Hokkaido University Animal Research Committee and performed in a BSL3 facility. Virus isolation from lung tissue was attempted 3 months after the inoculation by inoculation to tissue culture cells as described previously [25]. Briefly, the lung tissue homogenate was cultivated on a Vero E6 cell monolayer, and after 1 week, cells were harvested and

Table 2. FRNT titers and IgM antibodies of IgG positive rats capture in Saigon Harbor.

| Serum ID | FRNT titers against | | | IgM |
|---|---|---|---|---|
| | SR-11 | CSG5 | THAIV | SR-11 |
| CSG5/07 | <40 | NT* | <40 | + |
| CSG11/07 | 80 | NT | <40 | – |
| CSG41/07 | 320 | NT | <40 | – |
| CSG45/07 | 320 | NT | <40 | – |
| CSG58/07 | 640 | NT | <40 | – |
| CSG60/07 | 640 | NT | <40 | – |
| CSG19/08 | 2560 | 640 | NT | NT |
| CSG24/08 | 640 | 320 | NT | NT |
| CSG25/08 | 160 | 320 | NT | NT |
| CSG40/08 | 320 | 320 | NT | NT |
| CSG51/08 | 320 | 640 | NT | NT |
| anti-SEOV** | 80 | 80 | <40 | – |
| anti-THAIV*** | <40 | <40 | 80 | – |
| Negative rat | <40 | <40 | <40 | – |

* Not tested.

Rat sera experimentally inoculated with SEOV strain SR-11** or THAIV strain Thai749*** were previously prepared (8) (18).

cell smears on a glass plate were fixed with acetone. Viral antigens in the cells were detected by IFA with Alexa488-labeled E5/G6 monoclonal antibody against N protein of HTNV as described previously [12]. Culture supernatant of Vero E6 cells, which were positive by IFA, was stocked at −80°C as seed virus.

## RESULTS

*Serological examination in rats*: Thirty (2.8%) of the 1,066 serum samples from *Rattus* species were found to be positive against HTNV strain 76–118 or SEOV strain SR-11 antigens by all three tests, ELISA, IFA and Western blotting, and were regarded as serologically positive in hantavirus infection (Table 1). Positive rodents were obtained only from *Rattus norvegicus*. Positive rats were obtained from locations at Saigon Harbor (23/111, 20.7%), urban area of HCMC District 12 (5/16, 31.3%) and Dong Nai Province (2/46, 4.3%), but not in Tra Vinh Province, Vinh Long Province or Central Highland area (Table 1 and Fig. 1).

*Virus isolation from rats*: To select suitable lung tissues for virus isolation, a total of 11 serum specimens were selected from 23 positive sera from *Rattus* captured at Saigon Harbor based on the volume of serum specimens and availability of lung tissues. FRNT titers were measured in the 11 selected sera against SEOV strain SR-11 and THAIV strain Thai749 (Table 2). All of the sera except CSG5/07 showed FRNT titers against SR-11 from 1:80 to 1:2,560. Six serum samples showed undetectable levels of FRNT titer (<40) to THAIV strain Thai749. These 6 serum samples were further examined by ELISA for IgM antibody titers to strain SR-11. Only one serum sample, CSG5/07, was found to be positive for IgM antibody to strain SR-11. Since serum specimen CSG5/07 possessed no neutralizing antibody but possessed IgM antibody, this rodent was considered to be in the acute phase of infection. Therefore, we selected lung tissue from corresponded rat, rat #5, captured at Saigon Harbor in 2007. We also selected one additional lung tissue from corresponded rat of serum CSG11/07 as the neutralizing antibody titer was low (1:80), that is from rat #11 captured at Saigon Harbor in 2007.

Mice inoculated with lung materials from rat #5, began to show clinical symptoms, such as ruffled fur and weight loss, at 3 months after inoculation. Lung materials from rat #11 caused no change in mice. The lung homogenate of the mice inoculated with lung material from rat #5 was inoculated in Vero E6 cell culture, and finally hantavirus was isolated. The virus was designated as strain CSG5.

A total of 5 serum specimens obtained from antibody-positive rats captured at Saigon Harbor and immune sera against SEOV strain SR-11 showed FRNT titers against CSG5, with the same titers or within two-fold difference in titers against SR-11 and CSG5 except for one serum sample (CSG19/08) (Table 2). FRNT titer against THAIV strain Thai749 was under detection limit.

*RT-PCR test*: A total of 16 lung specimens were selected from antibody-positive rats captured at Saigon Harbor (13 rats) or District 12 of HCMC (3 rats) and subjected to RT-PCR. All of the specimens except that from CSG41 were found to be positive for S and M genome segments of the hantavirus. Only M segment was amplified from the specimen from CSG41. Figure 2A shows a phylogenetic tree based on the full-length S segment sequence. Sequences obtained from rats captured at Saigon Harbor and District 12 of HCMC belonged to the same lineage as SEOVs obtained from rats captured at Hai Phong Port and Singapore. Virus sequences obtained from rats captured at Saigon Harbor were found to be mixed with Northern viruses (Haiphong port) and suburban viruses. In the phylogenetic tree based on partial M segments, all of the Vietnam viruses were clustered within the same clade (Fig. 2B). Phylogenetic trees based on the partial S segment nucleotide sequences also showed that

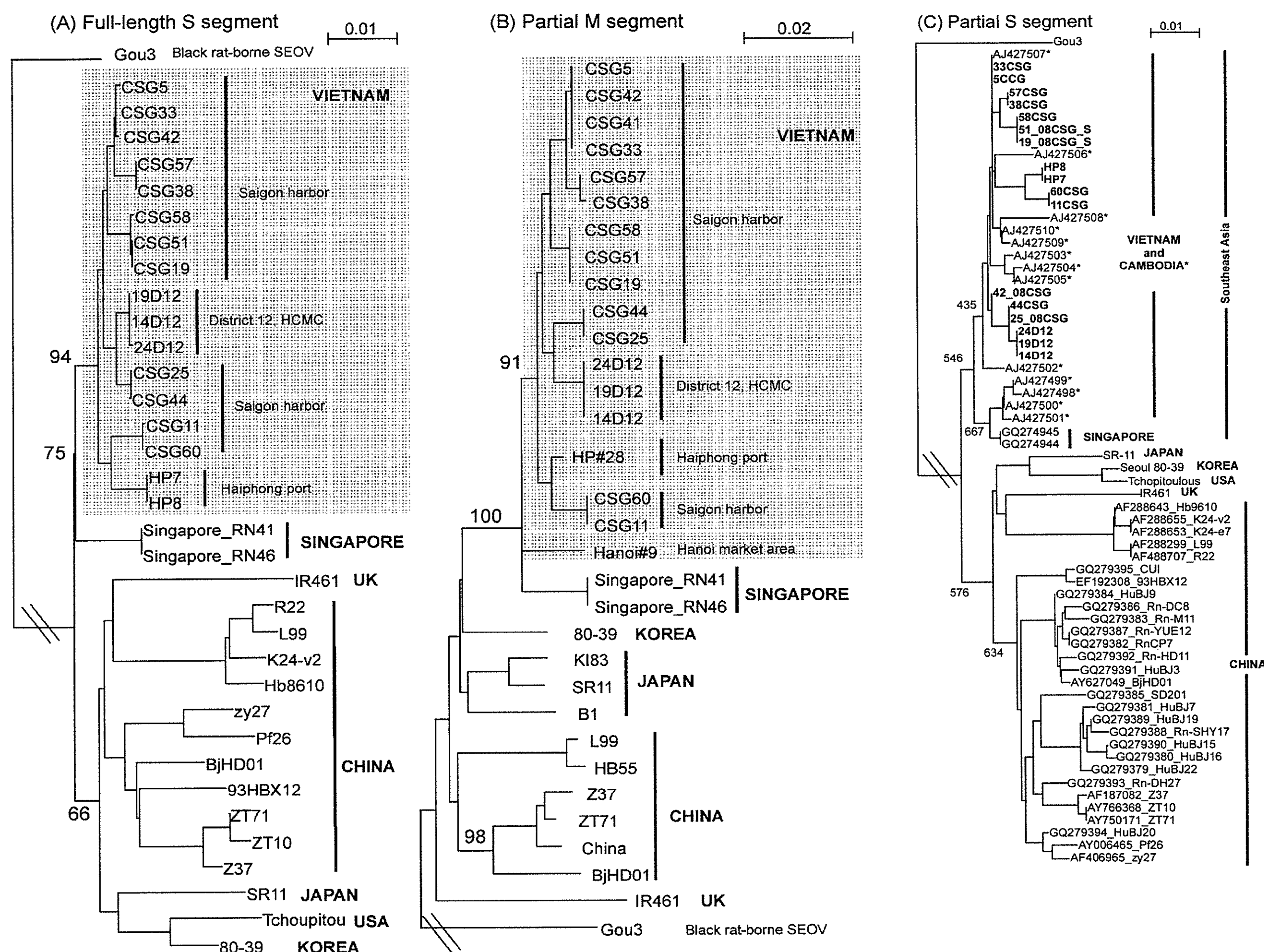


Fig. 2. Phylogenetic analysis of Vietnamese hantaviruses.
A: Phylogenetic analysis of hantaviruses based on full-length S segment sequence. Sequences of SEOV strains Gou3 (AF184988), HP7 (AB618143), HP8 (AB618144), IR461 (AF329388), R22 (AF288295), L99 (AF288299), K24-v2 (AF288655), Hb8610 (AF288643), zy27 (AF406965), Pf26 (AY006465), BjHD01 (AY627049), 93HBX12 (EF192308), ZT71 (AY750171), ZT10 (AY766368), Z37 (AF187082), SR-11 (M34881), Tchoupitoulas (AF329389), Seoul 80-39 (NC005236), Singapore_RN41 (GQ274944), and Singapore_RN46 (GQ274945) were used. The sequences of Vietnamese SEOV derived from Rattus norvegicus captured at Saigon Harbor (CSG5, CSG11, CSG19, CSG25, CSG33, CSG38, CSG42, CSG44, CSG51, CSG57, CSG58, and CSG60) and in a suburban area of HCMC, district 12 (14D12, 19D12, and 24D12) correspond to AB618112-AB618123 and to AB618124-AB618126, respectively.
B: Phylogenetic analysis of hantaviruses based on 1101 nt from the Gc region. Sequences of SEOV strains Gou3 (AB027521), HP#28 (AB355731), Hanoi#9 (AB355732), Singapore_RN41 (GQ274942), Singapore_RN46 (GQ274943), Seoul 80-39 (S47716), KI83 (D17592), SR11 (M34882), B1 (X53861), L99 (AF035833), HB55 (AF035832), Z37 (AF190119), ZT71 (EF117248), China (EU163437), BjHD01 (DQ133505), and IR461 (AF458104) were used. The sequences of Vietnamese SEOV derived from Rattus norvegicus captured at Saigon Harbor (CSG5, CSG11, CSG19, CSG25, CSG33, CSG38, CSG41, CSG42, CSG44, CSG51, CSG57, CSG58, and CSG60) and in a suburban area of HCMC, district 12 (14D12, 19D12, and 24D12) correspond to AB618130 – AB618142 and to AB618127 – AB618129, respectively.
C: Phylogenetic analysis of hantaviruses based on 585 nt from the S segment. Cambodian virus sequences derived from R. norvegicus, AJ427498 – AJ427510 were used. In addition to the strains used in B, numerous SEOVs from China were included. Their accession numbers are shown in the Figure. Gou3 was used as an outgroup.

Vietnamese and Cambodian SEOVs are placed into a mixed lineage (Fig. 2C). Thus, South East Asian hantaviruses were found to be different from China and Far East strains.

*Serological examination in the shrew*: Thirty-two of the 245 shrew serum samples were positive against TPMV antigen by all three tests, ELISA, IFA and Western blotting. All except 4 of the shrew serum samples were also positive by FRNT (Table 3). Thus, we considered the shrews to be TPMV- or antigenically related virus-infected shrews. Positive shrews were obtained from the Central Highland area and a rubber farm in Don Nai Province (Table 1 and Fig. 1). The mitochondrial *cyb* gene sequences (600 bp) of six shrews captured in the Central Highland area Gia Lai province were identical to the published *cytb* sequence of *S.*

Table 3. FRNT titers of shrew sera against TPMV

| Serum ID | Year | Origin | FRNT titers |
|---|---|---|---|
| #46 | 2006 | Dong Nai (farm) | 40 |
| #60 | 2006 | Dong Nai (farm) | 80 |
| #141 | 2007 | Chuse-Gia lai | 80 |
| #277 | 2007 | Chuse-Gia lai | 40 |
| #300 | 2007 | Kontum | 80 |
| #306 | 2007 | Kontum | 160 |
| #310 | 2007 | Kontum | 160 |
| #312 | 2007 | Kontum | 160 |
| #322 | 2007 | Kontum | 40 |
| #324 | 2007 | Kontum | 80 |
| #384 | 2007 | Pleiku-Gia lai | 80 |
| #415 | 2007 | Pleiku-Gia lai | 640 |
| #419 | 2007 | Pleiku-Gia lai | 320 |
| #421 | 2007 | Kontum | 80 |
| #425 | 2007 | Kontum | 160 |
| #433 | 2007 | Kontum | 320 |
| #54 | 2008 | Pleiku-Gia lai | 40 |
| #58 | 2008 | Daklak | 40 |
| #39 | 2009 | Gia lai | <40 |
| #41 | 2009 | Gia lai | 320 |
| #42 | 2009 | Gia lai | 160 |
| #68 | 2009 | Gia lai | <40 |
| #81 | 2009 | Gia lai | <40 |
| #84 | 2009 | Gia lai | 40 |
| #94 | 2009 | Gia lai | 320 |
| #456 | 2009 | Gia lai | 2560 |
| #458 | 2009 | Gia lai | 640 |
| #459 | 2009 | Gia lai | 40 |
| #461 | 2009 | Gia lai | 160 |
| #462 | 2009 | Gia lai | <40 |
| #463 | 2009 | Gia lai | 320 |
| #464 | 2009 | Gia lai | 1280 |
| Immune Shrew | - | Laboratory shrew | 160 |
| Negative shrew | - | Laboratory shrew | <40 |

*murinus*(FJ813963).

## DISCUSSION

In this study, we examined the distribution of SEOV infection among *Rattus* species in a port area and neighboring urban areas in Southern Vietnam. The results obtained confirmed and extended the results of our previous study mainly conducted in Haiphong Port, Hanoi City and neighboring provinces in Northern Vietnam [22], where SEOV infection was confirmed among *R. norvegicus*. These results strongly suggest that SEOV infection is distributed throughout Vietnam including port areas and urban areas.

In this study, strain CSG5 that was confirmed to be SEOV serologically and genetically was isolated from lung tissue of a rat captured at Saigon Harbor in 2007. Phylogenetic analysis showed that strain CSG5 was placed in the same lineage as virus sequences obtained from rats captured at Haiphong Port in Northern Vietnam [22]. Additional sequences of amplicons from rats captured at Saigon Harbor and District 12 of an urban area in HCMC were also placed in the same lineage. These results indicated that SEOV infection might have spread among rats not only in port areas but also in inland large cities throughout Vietnam. Although serological surveillance among humans in Southern Vietnam is still limited, an HFRS case that was infected with SEOV was reported in HCMC [5]. Thus, further consideration of the status of hantavirus infection is necessary from a public health point of view.

Phylogenetic characterization based on nucleotide sequences of the partial S segment of SEOV isolated in South East Asian countries including Cambodia [18] and Singapore [6] indicated that they formed one South East Asian virus lineage. Our previous study on phylogenetic characterization of many SEOVs isolated in China, Japan, Korea and U.S.A. showed that they were divided into 5 closely related subgroups within one clade and that viruses in one subgroup

seemed to have migrated to East Asian countries and U.S.A. [23]. The present study showed that the clade with South East Asian viruses was clearly different from the clade with China and East Asian viruses.

Rodents in the genus *Rattus*, which are now distributed worldwide, are thought to have originated in South East Asia because most *Rattus* species were found in this area [11]. Therefore, it is speculated that the ancestral SEOV moved from South East Asia to East and North East Asia with the migration of ancestral *Rattus* species.

Thottapalayam virus (TPMV) antibody-positive Asian house shrews were obtained in the Central Highland area. The positive sera were able to neutralize infection with TPMV, which is a prototype virus of shrew-born hantavirus. Therefore, these results indicated that TPMV or a virus antigenically related to TPMV exists among shrews in the Central Highland area in Vietnam. Since positive rates are high in several areas, TPMV might be endemic among shrews in Vietnam (Table 1). So far, a febrile patient having anti-TPMV antibody in northern Thailand and two antibody-positive *Suncus murinus* in Indonesia have been reported [13]. Although epidemiological and epizootiological observations are limited, the results obtained in Vietnam and other Asian countries strongly suggest that TPMV infection is spreading in East Asian countries. So far, we have not succeeded in virus isolation and RT-PCR from shrew specimens. We used primer sets based on prototype TPMV sequence (data not shown). We speculated that these primers were not adequate to amplify Vietnamese TPMV viruses. Recently, variation of TPMV was reported from China [4]. This finding might help primer setting to detect Vietnamese TPMV variants.

In conclusion, we have found that various hantaviruses might exist in East to South East Asian countries. Further epidemiological and epizootiological studies are necessary to clarify the variation of hantaviruses in more detail as well as the possible role for human pathogens.

ACKNOWLEDGMENTS. This study was supported in part by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT, Japan. This work was also supported, in part, by a grant from the Global COE program (Establishment of International Collaboration Center for Zoonosis Control) and also supported in part by Grants-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare including H22-emerging-ippan-006.

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Arch Virol (2012) 157:2179–2187
DOI 10.1007/s00705-012-1405-9

ORIGINAL ARTICLE

# Novel serological tools for detection of Thottapalayam virus, a Soricomorpha-borne hantavirus

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Received: 26 January 2012 / Accepted: 25 May 2012 / Published online: 25 July 2012
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**Abstract** We developed serological tools for the detection of hantavirus-specific antibodies and hantavirus antigens in shrews. The work was focussed to generate Thottapalayam virus (TPMV)-specific monoclonal antibodies (mAbs) and anti-shrew immunoglobulin G (IgG) antibodies. The mAbs against TPMV nucleocapsid (N) protein were produced after immunization of BALB/c mice with recombinant TPMV N proteins expressed in *Escherichia coli*, baculovirus and *Saccharomyces cerevisiae*-mediated expression systems. In total, six TPMV N-protein-specific mAbs were generated that showed a characteristic fluorescent pattern in indirect immunofluorescence assay (IFA) using TPMV-infected Vero cells. Out of the six mAbs tested, five showed no cross-reaction to rodent-associated hantaviruses (Hantaan, Seoul, Puumala, Tula, Dobrava-Belgrade and Sin Nombre viruses) in IFA and enzyme-linked immunosorbent assay (ELISA), although one mAb reacted to Sin Nombre virus in IFA. None of the mAbs cross-reacted with an amino-terminal segment of the shrew-borne Asama virus N protein. Anti-shrew-IgG sera were prepared after immunization of rabbits and BALB/c-mice with protein-G-purified shrew IgG. TPMV-N-protein-specific sera were raised by immunisation of Asian house shrews (*Suncus murinus*) with purified yeast-expressed TPMV N protein. Using these tools, an indirect ELISA was developed to detect TPMV-N-protein-specific antibodies in the sera of shrews. Using an established serological assay, high TPMV N protein specific antibody titres were measured in the sera of TPMV-N-protein-immunized and experimentally TPMV-infected shrews, whereas no cross-reactivity to other hantavirus N proteins was found. Therefore, the generated mAbs and the established ELISA system represent useful serological tools to detect TPMV, TPMV-related virus antigens or hantavirus-specific antibodies in hantavirus-infected shrews.

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## Introduction

Hantaviruses, which are members of the family *Bunyaviridae*, genus *Hantavirus*, are negative-sense, single-stranded RNA viruses with three segments, designated large (L), medium (M), and small (S), which encode an RNA-dependent RNA polymerase, two envelope glycoproteins (G1/Gn and G2/Gc) and a nucleocapsid (N) protein [11]. Rodent-borne hantaviruses are the causative agents of two viral zoonoses: haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). In general, each hantavirus species is associated with a single predominant mammal species or a closely related species of the same genus that serves as its natural reservoir. This close relationship and the congruencies of phylogenies between hantaviruses and their hosts, could be explained by a virus-host co-evolution or, alternatively, by host-switching events followed by host adaptation processes [31, 32, 36].

Thottapalayam virus (TPMV), which was isolated from an Asian house shrew (order Soricomorpha, family Soricidae, *Suncus murinus*) that was captured during a survey for Japanese encephalitis virus in Tamil Nadu, India in 1964, for a long time represented the only exception of a non-rodent associated hantavirus [5]. Only recently was the complete genome of TPMV determined and the host association with *S. murinus* confirmed [38]. Recently, in addition to TPMV, numerous shrew- and mole-associated hantaviruses have been reported from America, Europe, Asia and Africa [1, 2, 12–14, 16, 37, 39, 40, 46]. These investigations were facilitated by the development of a broad-spectrum pan-L RT-PCR assay [15]. Previous investigations were mainly based on RT-PCR approaches. Therefore, little is known about the pathogenicity, the course of infection, or the humoral immune response in infected hosts. Moreover, the antigenic characteristics of TPMV and the new Soricomorpha-borne hantaviruses and their differences to those of rodent-borne hantaviruses have not yet been investigated. As evidenced by nucleotide (nt) and amino acid (aa) sequence analysis of the full-length S, M, and L segments and the encoded proteins, TPMV is the most genetically divergent from all other hantaviruses [38, 43]. In addition, either very low or no antigenic cross-reactivity has been observed between TPMV and other hantaviruses [6]. The current serological detection system for TPMV-specific antibodies in human and shrew sera is based on a monoclonal antibody (mAb) E5/G6 capture enzyme-linked immunosorbent assay (ELISA) using a recombinant TPMV N fusion protein with an E5/G6 epitope. Using this assay, a TPMV infection in a Laotian immigrant with a febrile illness was detected, and anti-TPMV antibodies in two out of 14 Asian house shrews captured in Indonesia were also detected [30].

For rodent-borne hantaviruses, a large panel of N- and glycoprotein-specific mAbs has been developed [3, 7, 8, 10, 17–24, 26, 34, 35, 41, 45, 49–51], but none are available for Soricomorpha-associated hantaviruses. In addition, immunological or serological investigations in shrews are also limited by the lack of commercially available species-specific secondary antibodies. For these reasons, our study was focussed on two areas: generation of TPMV-specific mAbs for detection of TPMV in infected cells and development of anti-shrew IgG antibodies applicable as species-specific secondary antibodies in immunological assays. The generation of the TPMV antigen using three different expression systems should prove to be valuable for generating TPMV-specific mAbs that are able to recognize the native antigen in virus-infected cells.

## Materials and methods

### Generation of recombinant full-length and truncated N proteins in *Escherichia coli* and insect cells

The full-length recombinant N protein of TPMV (accession no. AY526097) was expressed previously in both *E. coli* and insect cells using a baculovirus vector [30]. To construct plasmids expressing truncated N proteins spanning aa 1-80, 1-177, and 1-311 in *E. coli*, the corresponding coding regions were amplified by PCR using the following primers (restriction sites are given in capital letters): forward primer TPMVNATG#637, 5′-ttc aGA ATT Cga tga ctc aag gga aaa tga ctc ccg aag a; reverse primers TPMV290, 5′-ggC TCG Aga gca agc ata gct tgc ccg g, TPMV580, 5′-atC TCG AGg tcc tcc atg cat gag tca t; TPMV980, 5′-aaC TCG AGt ggg gtg gct tct gac tca a. To express a truncated N protein of shrew-borne Asama virus (ASAV; accession number EU929070), a region of the S segment encoding the amino-terminal 103 aa of the N protein was amplified by PCR using the primer pair ASAVs-ATG-EcoR I (5′-ata GAA TTC atg gac aac att gag gac atcc) and ASAVs-tr-NP-103-Xho I-TAG (5′-ata CTC GAG cta gat tgt att acc ata ccg c). The TPMV- and ASAV-derived PCR products that were obtained were ligated to the plasmid vector pET43.1b (Novagen, Merck, Darmstadt, Germany). The recombinant expression plasmids were used to transform competent BL21 cells (Invitrogen, Life Technologies, New York, USA). Synthesis of recombinant proteins was induced by the addition of isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG: Wako, Osaka, Japan). Truncated N proteins fused to Nus-tag (491 amino acids) were extracted from *E. coli* and purified using a His-trap column according to the instructions of the manufacturer (Amersham, GE Healthcare Bio-Sciences, New York,

USA). An attempt to express the entire ASAV N protein in *E. coli* failed (data not shown).

Construction of a yeast expression plasmid and yeast expression of TPMV and other hantavirus N proteins in *Saccharomyces cerevisiae*

The entire open reading frame of the TPMV N protein was amplified by PCR using plasmid pFastBac–TPMV N [30] as a template with primers TPMV-5′ (5′-gat TCT AGA act caa ggg aaa atg act ccc gaa gag-3′) and TPMV 3′ (5′-gat TCT AGA tta cag ttt aat agg ctc ctg act tga-3′), which resulted in the addition of XbaI restriction sites to facilitate subsequent cloning. The XbaI-digested PCR product was cloned into XbaI-linearized yeast expression plasmid pFX7-His6 [33]. DNA sequence determination revealed a few nt exchanges compared to the published sequence (accession no. AY526097), which led to four aa exchanges (Q20K, A102T, G346S, G357A). For expression, *S. cerevisiae* strain gcn2 was transformed with a pFX7-derived expression plasmid encoding the TPMV N protein (pFX7-His6-TPMV N). Cultivation of yeast cells and expression and purification of TPMV, Tula virus (TULV), Puumala virus (PUUV) and Dobrava-Belgrade virus (DOBV) N proteins were performed as described previously [27, 28, 33].

Preparation of TPMV-N-protein specific monoclonal antibodies

Female BALB/c mice (Japan SLC, Inc, Hamamatsu, Japan and Taconic Europe, Ry, Denmark) were immunized four times with different recombinant TPMV N protein antigens (Table 1). Three days prior to fusion, the mice received a final immunization, and spleen cells from immunized mice were fused with myeloma P3X63Ag8U1 or SP2/0 cells using PEG1500 (Sigma-Aldrich, St. Louis, USA). Hybridoma supernatants were screened for TPMV-N-protein-specific antibodies in IFA and ELISA. Positive clones were re-cloned twice and TPMV-specific mAbs were further characterized as described below. The mAbs EB5, ED5, 1A3 and B5H9 were selected for subtyping in cell culture supernatant by ELISA using peroxidase-conjugated rabbit anti-mouse IgG-1, IgG-2a, IgG-2b, IgG-3, IgM, IgG (Zymed, Vienna, Austria), Lambda and Kappa (Caltag, Buckingham, UK) (1:5000). The mAbs 2H6 and 1F1 were subtyped using a Mouse Monoclonal Subtyping Kit (AbD Serotec, Kidlington, UK).

Indirect immunofluorescence assay (IFA)

In-house IFAs were performed essentially as described previously [47]. Acetone-fixed, TPMV, HTNV, SEOV, PUUV, TULV, or DOBV-infected Vero cells were used as antigens. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Zymed), was used as a secondary antibody. Commercial IFAs (Euroimmun, Lübeck, Germany) were performed according to the manufacturer's instructions using FITC-conjugated anti-mouse Ig (DakoCytomation, Glostrup, Denmark) as a secondary antibody.

**Table 1** Characteristics of monoclonal antibodies produced in this study

| Clone ID | Expression system used for generation of immunogen | Class/ subclass | ELISA detection with screening antigen |
|---|---|---|---|
| EB5 | Baculovirus | IgM | *E. coli*-expressed TPMV N protein |
| ED5 | Baculovirus | IgM | *E. coli*-expressed TPMV N protein |
| 1A3 | *Escherichia coli* | IgG1/kappa | *E. coli*-expressed TPMV N protein |
| B5H9 | *Escherichia coli* | IgG* | *E. coli*-expressed TPMV N protein |
| 2H6 | *Saccharomyces cerevisiae* | IgG1/kappa | Yeast-expressed TPMV N protein |
| 1F1 | *Saccharomyces cerevisiae* | IgG1/kappa | Yeast-expressed TPMV N protein |

Enzyme-linked immunosorbent assay, ELISA; Ig, immunoglobulin; N, nucleocapsid; TPMV, Thottapalayam virus

* The subclass and light chain of B5H9 were not defined

Enzyme-linked immunosorbent assay (ELISA)

Flat-bottomed 96-well plates were coated with purified *E. coli* or insect-cell-expressed TPMV and ASAV N proteins in phosphate-buffered saline (PBS), or with *S. cerevisiae* expressed TPMV, TULV, PUUV and DOBV N proteins in 0.05 M carbonate buffer and incubated overnight at 4 °C. PBS with 0.05 % Tween 20 containing 3 or 1 % bovine serum albumin (Sigma-Aldrich, St. Louis, USA) was added to block unsaturated binding sites, and the plates were incubated for 1 h at 37 °C or room temperature. After washing three times with 0.05 % PBS-Tween, hybridoma supernatants were added, and the plates were incubated for 1 h at 37 °C. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000) and *o*-phenylendiammonium dichloride substrate (Sigma-Aldrich, St. Louis, USA) or 3,3′,5,5′-tetramethylbenzidine (Bio-Rad, Hercules, California, USA). After incubating for 15 min at room temperature, the absorbance at 450 nm was measured.

Western blot test

The reactivity of the mAbs with full-length or truncated recombinant N proteins of TPMV and ASAV was tested in

a western blot test (WB) as described previously [48]. The purified yeast-expressed TPMV N protein was tested in the WB with mAbs raised against PUUV (2C6, 7A5, 5E11, 5C5, [52]; A1C5, [51]; 4C3, 2E12, 5A3, 1C12, [25]), SNV/ANDV (7G2, 4H3, [18]) and HTNV (E5/G6, ECO2, [49]; B5D9, [51]). The synthesis of the Nus fusion proteins was confirmed in the WB by staining with anti-NUS-tag mAb (Novagen).

### Purification and biotinylation of the immunoglobulin fraction

The mAbs ED5 (IgM) and 1A3 (IgG) were purified using an IgM purification kit (Pierce, Thermo Scientific, Rockford, Illinois, USA) using protein-A Sepharose column chromatography and a MAPS II kit (Bio-Rad), respectively. The purified mAbs were biotinylated using a Biotin-AC5-OSu kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

### Competitive binding to *E. coli*-and insect-cell-expressed TPMV N proteins in ELISA

The relative binding avidity of mAbs to solid-phase recombinant antigen was evaluated by indirect ELISA. Flat-bottomed 96-well microtitre plates were coated with serial twofold dilutions of purified truncated N protein in PBS and then incubated overnight at 4 °C. Nonspecific binding was blocked with PBS containing 3 % bovine serum albumin for 1 h at room temperature. The plates were then washed three times with 0.05 % PBS-Tween. Dilutions of each biotin-labeled mAb were added and incubated for 1 h at room temperature. After additional washing, HRP conjugate (1:2000; Prozyme, Hayward, Canada) was added to the wells and incubated for 1 h at room temperature. Thereafter, *o*-phenylendiammonium dichloride substrate was added, and the plates were incubated for 30 min at room temperature. The relative binding avidity was defined as the amount of antibody required to yield an A450 value ranging from 0.4 to 1.4. Absorbance values at 405 nm were compared with those in the absence of a competitive antibody. For use in competitive binding assays, the serial fivefold dilutions of each unlabeled antibody were added to 96-well plates coated with antigen as described above. After a one-hour incubation at room temperature, the plates were washed, and binding of biotinylated mAbs was detected with avidin-peroxidase as described earlier.

### Establishment of an ELISA to detect TPMV-specific antibodies in shrew sera

To establish this diagnostic tool, anti-shrew-IgG antisera were first prepared. Briefly, two BALB/c mice and two rabbits were immunized five times at four-week intervals with 200 µg protein-G-purified shrew IgG. To obtain shrew anti-TPMV-N sera, three Asian house shrews were immunized four times at four-week intervals with 50 µg of dialyzed TPMV N protein, and blood was taken after each immunization. In addition, two Asian house shrews were inoculated intraperitonially with $10^4$ FFU of TPMV (Arikawa et al. unpublished data).

To test the shrew anti-TPMV-N-protein antisera, the anti-shrew-IgG, and the sera from experimentally TPMV-infected shrews, an indirect cross-titration ELISA was performed. Briefly, plates were coated as described earlier. The different shrew sera were tested on plates coated with TPMV, TULV, PUUV or DOBV N protein. As secondary antibodies, the anti-shrew-IgG antisera were cross-titrated. To determine the amount of antibody bound, an anti-mouse-IgG HRP-conjugate was used as a third antibody. All incubations were done for 1 h at 37 °C, and between the single incubation steps, the plates were washed three times as described above.

## Results

For the generation of TPMV-N-protein-specific mAbs, the entire N protein of 435 aa residues was expressed using *E. coli* and baculovirus-mediated insect cell systems [30] and a yeast expression system. The high-level yeast expression of the entire His-tagged TPMV N protein resulted in a protein of the expected molecular weight (data not shown). This purified protein did not react in the immunoblot with any of the PUUV-specific mAbs (2C6, 7A5, 5E11, 5C5, A1C5, 4C3, 2E12, 5A3, 1C12), the SNV/ANDV-specific mAbs (7G2, 4H3) or the HTNV-specific mAbs E5/G6, ECO2, B5D9 (data not shown).

Six different mAbs were generated that recognized the TPMV N protein expressed in *E. coli*, insect cells and *S. cerevisiae*, and these were characterized by ELISA, IFA and WB (Tables 1, 2). The subtyping of the generated mAbs showed that the clones EB5 and ED5 were of the IgM class, whereas the remaining clones belong to the IgG class (Table 1). All six mAbs showed a characteristic cytoplasmic fluorescent pattern in IFA when using an acetone-fixed smear of TPMV-infected Vero cells and recombinant TPMV N protein expressed in insect High Five cells (Table 2). Their specificity was confirmed in ELISA and IFA against N proteins from rodent-associated hantaviruses (Hantaan virus, HTNV; Seoul virus, SEOV; PUUV; TULV; DOBV; Sin Nombre virus, SNV). Only the mAb ED5 was found to have cross-reactivity to SNV in IFA (Table 2). None of the TPMV-specific mAbs cross-reacted in the ELISA and WB with an amino-terminal segment of the shrew-borne ASAV N protein (Table 3).

**Table 2** Cross-reactivities of monoclonal antibodies with different hantaviruses in indirect immunofluorescence assay (IFA) using hantavirus-infected Vero cells and insect cells expressing hantavirus nucleocapsid (N) protein

| Clone ID | TPMV | | HTNV | SEOV | PUUV | TULV | SNV* | DOBV |
|---|---|---|---|---|---|---|---|---|
| | Infected Vero cells | N protein expressed in High Five cells | Infected Vero cells | | | | | |
| EB5 | + | + | – | – | – | – | – | – |
| ED5 | + | + | – | – | – | – | + | – |
| 1A3 | + | + | – | – | – | – | – | – |
| B5H9 | + | + | – | – | – | – | – | – |
| 2H6 | + | + | – | – | – | n.d. | – | – |
| 1F1 | + | + | – | – | – | n.d. | – | – |

+ positive, – negative, n.d. not done, TPMV Thottapalayam virus, HTNV Hantaan virus, SEOV Seoul virus, PUUV Puumala virus, TULV Tula virus, SNV Sin Nombre virus, DOBV Dobrava-Belgrade virus

* Recombinant N protein of SNV was expressed by baculovirus vector in High Five insect cells

**Table 3** Reactivities of monoclonal antibodies against *E. coli*-expressed full-length and truncated Thottapalayam virus (TPMV) and Asama virus (ASAV) nucleocapsid (N) proteins in ELISA and western blot test

| Clone ID | ELISA | | | | | Western blot test | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | TPMV N (aa) | | | | ASAV N (aa) | TPMV N (aa) | | | | ASAV N (aa) |
| | 1-80 | 1-177 | 1-311 | 1-435 | 1-103 | 1-80 | 1-177 | 1-311 | 1-435 | 1-103 |
| EB5 | – | – | – | + | – | – | n.d. | n.d. | – | – |
| ED5 | + | + | + | + | – | + | + | + | + | – |
| 1A3 | + | + | + | + | – | + | + | + | + | – |
| B5H9 | – | – | – | + | – | – | n.d. | n.d. | – | – |
| 2H6 | + | + | + | + | – | + | + | + | + | – |
| 1F1 | + | + | + | + | – | – | n.d. | n.d. | – | – |

aa amino acid position, + positive, – negative, n.d., not done

Four of the six mAbs that were generated were reactive in ELISA with *E. coli*-derived truncated TPMV N proteins including the amino-terminal 80 aa (Table 3). Three of these four mAbs (1A3, ED5 and 2H6) showed an identical pattern of reactivity in ELISA and WB (see Fig. 1; Table 3), whereas mAb 1F1 failed to detect the full-length and truncated N proteins in the WB. The two remaining mAbs, EB5 and B5H9, reacted with the full-length TPMV N protein in the ELISA but not in the WB. The relative binding avidity of the biotin-labeled mAbs (1A3, ED5), tested by avidin-biotin indirect ELISA, showed different competitive effects when tested with unlabeled mAbs to recombinant TPMV N protein (Fig. 2). Based on the different levels of competition, two groups were defined, one comprising mAbs 1A3 and ED5, and the other comprising EB5 and B5H9.

Purified shrew IgG from *S. murinus* was used to produce anti-shrew-IgG sera in two BALB/c mice and two rabbits. TPMV-N-protein-specific shrew control sera were produced in *S. murinus* by immunization with the yeast-expressed TPMV N protein. The specific titres of these

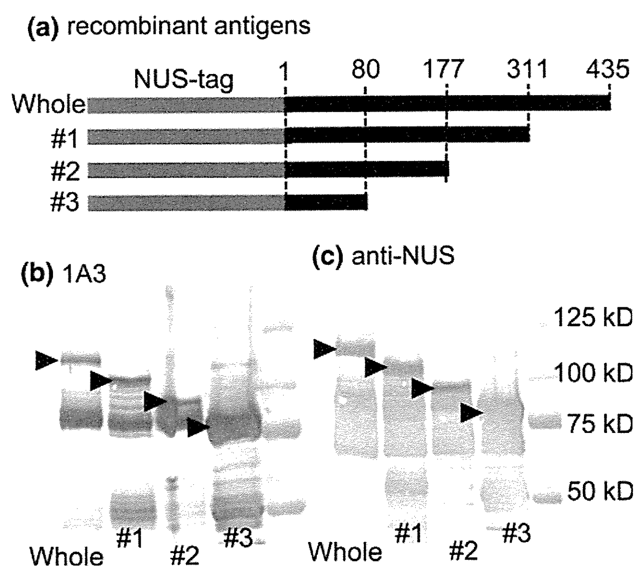


**Fig. 1** Schematic representation of the structure (**a**) and western blot reactivity of full-length and truncated Thottapalayam virus nucleocapsid protein derivatives with TPMV-specific monoclonal antibody (mAb) 1A3 (**b**) and anti-NUS mAb (**c**)

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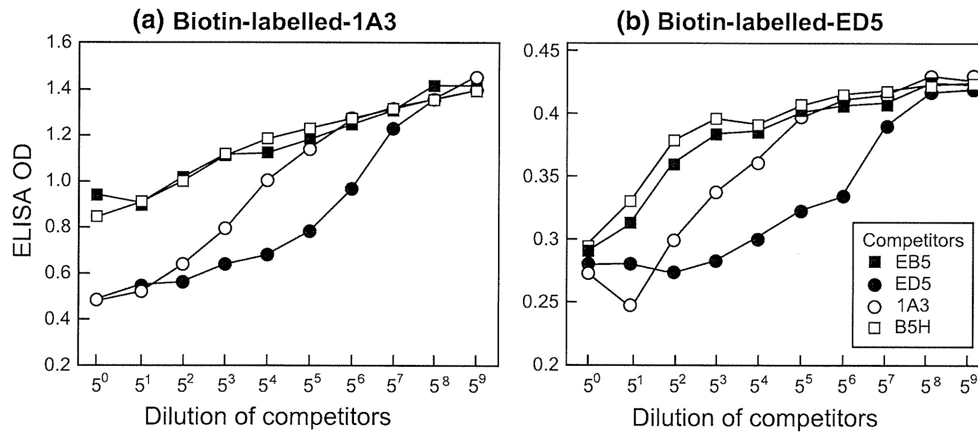


**Fig. 2** Competitive binding assay with biotin-labeled mAbs 1A3 (**a**) and ED5 (**b**). Absorbance values (OD) at 405 nm are shown on the y-axis and are compared with those in the absence of competitive antibody. Dilutions of the competitive antibodies are shown on the x-axis. The relative binding avidity was defined as the amount of antibody required to yield an A450 of 0.4–1.4. Purified mAbs (1 mg/ml) were diluted from 1:5 to 1:510 and added as competitors

antisera and of the sera of experimentally TPMV-infected shrews were determined by ELISA. Using an initial cross-titration ELISA, the optimal dilution of the anti-shrew IgG for detection of TPMV-N-protein-specific antibodies in an immunized shrew was selected to be 1:32,000. By using the mouse anti-shrew-IgG at this selected dilution, strong TPMV-specific ELISA reactivities were detected in sera from all three immunized shrews and the two infected shrews (Fig. 3 and data not shown). In addition, in all three shrews, TPMV-N-protein-specific antibodies were already detected after the second immunization (data not shown). The TPMV-N-protein specific antibody titre in one of the immunized animals reached a level of 1:128,000 12 days after the final, fourth immunization. In general, no cross-reactivity to other hantavirus N proteins was found in any

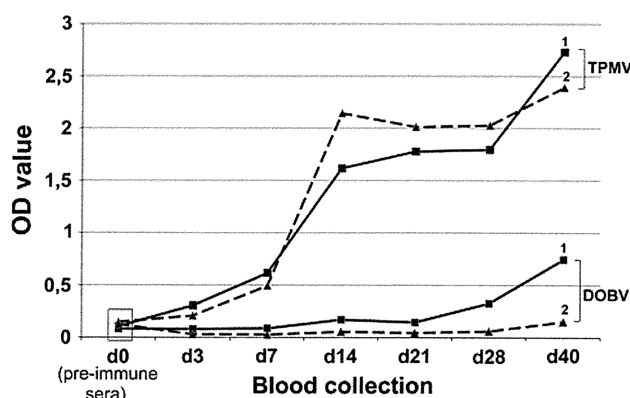


**Fig. 3** ELISA reactivity of the sera from two experimentally TPMV-infected Asian house shrews against yeast-expressed TPMV and DOBV N proteins. Absorbance values (OD) of the ELISAs measured at 405 nm are shown on the y-axis. Time points after the experimental infection are given on the x-axis. Reactivities of non-immunizied shrew sera (pre-immune sera) are highlighted by a box. d, days postinfection

of the three TPMV-immunized shrews (data not shown). Only in one of the infected animals (shrew 1), 40 days postinfection, during the high IgG antibody response against TPMV, was a low cross-reactivity against DOBV detected (Fig. 3).

## Discussion

Hantavirus-specific mAbs have been generated by various approaches [3, 10, 17, 19–24, 26, 34, 35, 41, 45, 49, 51]. Previously, yeast-expressed polyomavirus-derived virus-like particles harbouring 120 aa residues of the PUUV N protein and his-tagged N protein derivatives of SNV and ANDV were used for the generation of mAbs [18, 52]. These mAbs were found to react to the N antigens used for their generation, but also to a different extent to N proteins of other hantaviruses. Most importantly, these mAbs can be used to detect native viral antigen in virus-infected cell lines by IFA and in immunohistochemistry analysis of rodent and human tissue samples [18]. The TPMV-specific mAbs described here were also obtained by immunization with a His-tagged yeast-expressed TPMV N protein, confirming the value of this procedure. The generation of mAbs with similar properties using TPMV N protein from *E. coli* or baculovirus-mediated insect cell expression systems indicates the usefulness of all three different expression systems generating antigens for the production of mAbs. Moreover, the mAbs that were obtained, regardless of the expression system used for the generation of the antigen, were all found to detect native viral antigen in cell cultures.

The hantavirus N protein possesses immunodominant, linear and conformational cross-reactive epitopes within the first 100 aa of its N-terminus [9, 44, 49].

Mapping of the epitopes of N-protein-specific mAbs resulted in the identification of antigenic epitopes between aa 1-45 and 1-120 of PUUV, 166-175 of HTNV, 226-293 of TULV and 244-286 of ANDV and SNV [18, 24, 25, 42, 49]. In line with these observations, some of the TPMV-N-protein-specific mAbs described here are directed against the amino-terminal 80-aa region. The lack of reactivity of mAbs EB5 and B5H9 with the full-length N protein in the WB and with truncated N proteins in the WB and ELISA may suggest a conformation-dependent or discontinous epitope. Similarly, mAb 1F1 did not detect any TPMV N protein derivatives in the WB, suggesting that its binding site is at a conformational or discontinous epitope within the 80-aa amino-terminal region of the N protein. Future epitope mapping studies using linear synthetic peptides may allow localization of the linear epitopes of the mAbs ED5, 1A3 and 2H6 within the aa 1-80 region.

The lack of cross-reactivity of our mAbs to rodent-borne hantavirus N proteins (HTNV, SEOV, PUUV, TULV and DOBV) and ASAV N protein confirmed that TPMV is genetically and antigenetically highly divergent from all other known hantaviruses. In line with this observation, the yeast-expressed N protein of TPMV failed to react with a panel of mAbs raised against N proteins of PUUV, SNV/ANDV and HTNV. This particular feature of TPMV can be explained by the low aa sequence similarity of its N protein to those of other hantaviruses, which is also reflected in the isolated position of TPMV in phylogenetic trees [38, 43]. This lack of cross-reactivity is in contrast to the broad-spectrum cross-reactivity observed for rodent-borne hantavirus N-protein-specific mAbs with N proteins of these hantaviruses.

The mouse and rabbit anti-shrew-IgGs generated in this study are applicable in serological assays for the detection of IgG antibodies in sera of immunized and infected *S. murinus*, as demonstrated. Based on molecular data [29] and chromosome homology [4], *Suncus* represents a separated genus within the family Soricidae, which is closely associated with the genus *Crocidura*. The close relationship of these two genera, in spite of the detection of different new hantaviruses in members of the genus *Crocidura* [16, 40], may suggest that the application of the anti-shrew-IgG generated here is not limited to immunological approaches in *Suncus*. Indeed, an initial WB experiment demonstrated cross-reactivity of the rabbit anti-*Suncus* IgG with purified IgG of *Suncus*, *Crocidura* and *Sorex*, but a lack of reactivity with laboratory mouse IgG (data not shown). Additional efforts are needed to establish and validate serological assays to detect hantavirus-specific antibodies in these shrews by using defined negative and positive control sera. The efficiency of such serological assays could be improved by labelling the anti-shrew-IgGs with HRP, FITC or other fluorescent dyes.

In conclusion, the reactivity of the mAbs generated here in IFA using TPMV-infected cells indicates that these mAbs represent useful tools for detection of TPMV (and antigenically related hantaviruses) in cell culture and tissue of potentially infected animals or humans, where the shrew anti-TPMV-antisera could serve as a positive control. These serological tools will be helpful for discovering novel insectivore-associated hantaviruses and/or characterizing the humoral immune response and antigen expression in hantavirus-infected insectivores.

**Acknowledgments** The authors kindly acknowledge the support of Kathrin Heidemanns, Peter Giere and Swetlana Siniza and the critical reading of Daniel Balkema. The mAbs 2C6, 7A5, 5E11, 5C5, 7G2, 4H3, and 4C3, 2E12, 5A3, 1C12 were kindly provided by Aurelija Zvirbliene (Vilnius) and Åke Lundkvist (Stockholm). This study was supported in part by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, Ministry of Education, Culture, Sports, Science and Technology, Japan. This work was also supported in part by a grant from the Global COE program (Establishment of International Collaboration Centers for Zoonosis Control) and by Grants-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare. In addition, the study was partially funded by EU grant FP7-261504 EDENext and is catalogued by the EDENext Steering Committee as EDENext018 (http://www.edenext.eu). The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

**Ethical statement** Shrews, laboratory mice and rabbits were handled according to the Laboratory Animal Control Guidelines of the Hokkaido University Animal Research Committee in Japan and EU Council Directive 86/609/EEC for the protection of animals used for experiments in Germany.

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