

(PUUV), whose reservoir is *Myodes glareolus*, is the only known Arvicolinae rodent-associated causative agent of HFRS [21].

PUUV was discovered in the late 1970s and is the most common cause of HFRS in most European countries excluding the Mediterranean coast and northernmost areas [3]. Several thousands of cases are reported annually in Europe. In Russia and its neighboring countries, southern Siberia from the Ural Mountains to Lake Baikal, northern Kazakhstan, and areas of the Altai Sayan Mountains, *M. glareolus* harbors PUUV and causes HFRS [5].

Tula virus (TULV) was first discovered in tissue samples of the European common vole (*Microtus arvalis*) trapped in central Russia [18]. Subsequently, several other species of rodents of the subfamily Arvicolinae have been shown to have TULV in several countries where PUUV-infected *Myodes* also exist. Although TULV has been detected in various rodents throughout Europe, only two TULV-related HFRS cases have been reported to date [10,11,23]. These observations suggest that TULV is almost apathogenic in humans.

Several other Arvicolinae rodent-associated hantaviruses are listed in Table 1. Among them, those isolated from *Myodes rufocanus* (Hokkaido virus), *Myodes regulus* (Muju virus), *Microtus arvalis* (Tula virus), *Microtus pennsylvanicus* (Prospect Hill virus), *Microtus ochrogaster* (Bloodland Lake virus), *Microtus californicus* (Isla Vista virus), *Microtus fortis* (Vladivostok virus), *Microtus maximowiczii* (Khabarovsk virus), and *Lemmus sibiricus* (Topografov virus) are considered to be apathogenic because no human infections have been reported.

Various epidemiological and epizootiological studies have indicated different pathogenicities of *Myodes* spp.-derived PUUV and *Microtus* spp.-derived hantaviruses, which might be due to certain characteristics of the viruses.

To further characterize PUUV and TULV, we compared the antigenic cross-reactivities of PUUV and TULV using patient sera and immune sera obtained through animal experimentation.

2. Materials and methods

2.1. Animals

Japanese grass voles, *Microtus montebelli* (3–12 months old), bred and maintained in the laboratory, were purchased from Otsuka Pharmaceutical Company (Tokyo,

Japan). Before starting the experiment, voles were kept for 4 weeks with specific pathogen-free BALB/c mice (5–6 weeks old; Japan SLC, Inc., Hamamatsu, Japan) in the same cage as sentinel animals to monitor pathogens in the vole. The sentinel mice were clinically healthy during monitored periods and were shown to have no specific antibodies against mouse hepatitis virus, Mycoplasma, or Sendai viruses by the enzyme-linked immunosorbent assay (ELISA) diagnostic kit (Monilisa; Wakamoto Co., Tokyo, Japan) at the end of the observation period. No antibody to HTNV was detected by an indirect immunofluorescence antibody assay (IFA), as described below. All animals were handled according to the Laboratory Animal Control Guidelines of the Hokkaido University Animal Research Committee. Experimental infection of the virus was performed in a BSL3 facility.

2.2. Cells and viruses

The E6 clone of Vero cells (ATCC C1008 CRL1586) was grown in Eagle's minimal essential medium (MEM, 1× media; Invitrogen, Carlsbad, CA, USA) supplemented with Earle's salts, 2 mM L-glutamine (Invitrogen), 5% fetal calf serum, 0.5% nonessential amino acids, 5% insulin transferring selenium (ITS; Gibco, Grand Island, NY, USA), 100 mg/l streptomycin, and 10⁵ U/l penicillin at 37 °C. PUUV strain CG1820 and TULV strain Morabia were kindly provided by Dr. Alexander Plyusnin (Helsinki, Finland). The viruses were inoculated into Vero cells and cultured at 37 °C for 5 days. The culture supernatant was stored as a stock virus at –80 °C. The infectivity titers (focus-forming units, FFUs) of the stock viruses of PUUV and TULV were measured by counting infected cell foci detected by immunostaining as described previously with anti-Sin Nombre virus-N rabbit serum as the detecting antibody [1]. Infectivity titers of the PUUV and TULV stock viruses were 2 × 10⁴ and 1 × 10⁵ FFU/ml, respectively.

2.3. Experimental infection

Japanese grass voles (*M. montebelli*) were inoculated intraperitoneally (i.p.) with 1000 FFU of PUUV or 2000 FFU of TULV. Serum specimens and lungs were collected from two voles on days 4, 7, 10, 14, and 35 after inoculation. Serum antibodies against PUUV or TULV were examined by IFA or ELISA. The virus genome in the lung was assayed by RT-PCR as described in the following section.

Table 1
Hantaviruses carried by Arvicolinae rodents.

Rodent	Name	Distribution	Virus Species	Disease	References
<i>Myodes glareolus</i>	Bank vole	Europe	Puumala virus (PUUV)	HFRS	[3]
<i>Myodes rufocanus</i>	Gray red-backed vole	Japan, Far East Russia	Hokkaido virus (HOKV)	Apathogenic?	[9]
<i>Myodes regulus</i>	Korean red-backed vole	Korea	Muju virus (MUJV)	ND	[24]
<i>Microtus arvalis</i>	European common vole	Europe, Asia	Tula virus (TULV)	Apathogenic	[27]
<i>Microtus pennsylvanicus</i>	Meadow vole	North America	Prospect Hill virus (PHV)	Apathogenic	[13]
<i>Microtus ochrogaster</i>	Prairie vole	United States, Canada	Bloodland Lake virus (BLLV)	ND	U19303
<i>Microtus californicus</i>	California vole	Western United States	Isla Vista virus (ISLAV)	Apathogenic?	[25]
<i>Microtus fortis</i>	Reed vole	Far East Russia	Vladivostok virus (VLAV)	Apathogenic	[8]
<i>Microtus maximowiczii</i>	Maximowicz's vole	Far East Russia China	Khabarovsk virus (KBRV)	ND	[6] [32]
<i>Lemmus sibiricus</i>	Lemmings	Siberia	Topografov virus (TOPV)	ND	[28]

2.4. Sera

Nine sera from patients with HFRS in Germany were kindly provided by Dr. Rainer Ulrich, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute for Novel and Emerging Infectious Diseases, Germany. Sera confirmed to be infected with PUUV by a neutralization test [16] were used for evaluating the ELISA system established in this study. In addition, sera from three PUUV-like virus-infected gray red-backed voles (*Myodes rufocanus*) captured in Hokkaido, Japan, were kindly provided by Dr. Hiroaki Kariwa (Hokkaido University, Japan). As shown in Table 1, *M. rufocanus*-borne hantavirus is also recognized as Hokkaido virus (HOKV) distributed in the northern part of Japan and Far East Russia. HOKV was initially recognized as Japanese Puumala-like virus strains because of the high amino acid sequence similarities in the N protein to PUUV and the cross-neutralization pattern against European PUUV [9].

Immune mouse sera to PUUV and TULV were prepared in mice as follows. Specific pathogen-free BALB/c mice (females, 5–6 weeks old; Japan SLC, Inc.) were inoculated i.p. with 1000 FFU of PUUV (3 mice) or 2000 FFU of TULV (4 mice). Sera were collected at 35 days post-inoculation.

2.5. IFA slide preparation and testing

The IFA assay was performed as described previously [31]. Briefly, acetone-fixed smears of Vero E6 cells infected with PUUV or TULV were used as antigens. FITC-conjugated anti-mouse (Zymed, San Francisco, CA, USA) and FITC-conjugated Protein-A (EY Laboratories, San Mateo, CA, USA) were used for mouse and vole (*Microtus* spp. and *M. rufocanus*) sera, respectively [12]. The IFA antibody titer was regarded as the reciprocal of the highest serum dilution that displayed characteristic fluorescence in the infected Vero cells.

2.6. Expression of truncated N proteins of PUUV and TULV

Hantavirus N protein possesses an immunodominant antigenic region within the N-terminus of about 100 amino acids (aa) [4]. In this study, truncated N proteins of PUUV and TULV composed of aa 1–103 of the N-terminus were expressed using the pET43.1 plasmid vector (Novagen, Madison, WI, USA) and *Escherichia coli* host strain BL21, according to the manufacturer's instructions (Novagen pET System Manual 10th ed.). Briefly, the portion of the gene encoding 103 aa of the N protein of PUUV was amplified using primers PUUSwholeF (5'-cGAATTCatgagtactgacagacatccaa-3') and PUUS103R (5'-cccCTCGAGtcaatcaaggacatttccatc-3'). To amplify the corresponding region of TULV, primers TULS-F (5'-cGAATTCatgagccaactcaagaaatacaa-3') and TUL103-R (5'-cccCTCGAGtcaatcaaggacatttccatc-3') were used. A TGA stop codon was added to terminate recombinant protein translation (underlined). Fragments were cloned into the EcoRI/XhoI restriction site of the pET43.1 vector in-frame with the NUS and histidine (his)-tag, and the resultant

plasmids were introduced into *E. coli* host BL21 (DE3). Truncated NUS and his-tagged recombinant N proteins of PUUV and TULV were expressed in *E. coli* and then purified using HisTrap HP columns (Amersham, Piscataway, NJ, USA) according to the manufacturer's instructions. Purified antigens were evaluated by SDS-PAGE followed by protein staining (Simply blue; Invitrogen).

2.7. Establishment of ELISA

ELISA was carried out as described previously with some modifications. To determine the antigen concentration, flat-bottomed 96-well plates were coated with serial twofold dilutions of purified truncated N antigen in phosphate-buffered saline (PBS) and incubated overnight at 4 °C. After three washes with 0.05% Tween 20 in PBS (PBS-T), PBS containing 3% bovine serum albumin (Sigma Japan, Tokyo, Japan) was added to block unsaturated binding sites and incubated for 1 h at 37 °C. The plates were washed as above, an immune mouse sera (1:200) were added and incubated for 1 h at 37 °C. Bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000; Zymed) and *o*-phenyldiammonium dichloride substrate tablets (Sigma-Aldrich, St. Louis, MO, USA). After incubation for 15 min at room temperature, absorbance at 450 nm for each well was measured using a microplate reader (Spectramax 340; Molecular Devices, Sunnyvale, CA, USA). Finally, the optimal coating concentrations of truncated N antigens of both PUUV and TULV were determined.

Human sera were examined using essentially the same procedure as that described above to measure the antigen concentration with the following exceptions: antihuman IgG-alkaline phosphatase (1:2000; Sigma-Aldrich) and *p*-nitrophenyl phosphate tablets (Sigma-Aldrich) were used and absorbance values were measured at 405 nm. The patient sera were examined at 1:200 dilution.

Vole sera were examined using essentially the same procedure as above except with HRP-conjugated protein-A (1:200; Prozyme, San Leandro, CA, USA), *o*-phenyldiammonium dichloride substrate tablets (Sigma-Aldrich), and absorbance measurements at 450 nm.

2.8. RT-PCR

Total RNA was extracted from lung tissues of voles inoculated with hantaviruses using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RT-PCR was carried out as described previously [29]. Briefly, cDNA was synthesized from total RNA using SuperScript II (Invitrogen) and a random hexanucleotide primer. Nucleotide sequences of the virus S genome segment were amplified with two sets of primer pairs: GS4 (AGCTCIGGATCCATITCATC) and GS6 (GAIIGITGCCACCAACATG) for amplification of the PUUV S genome [2] and TULS-F and TUL103-R for amplification of the TULV S genome.

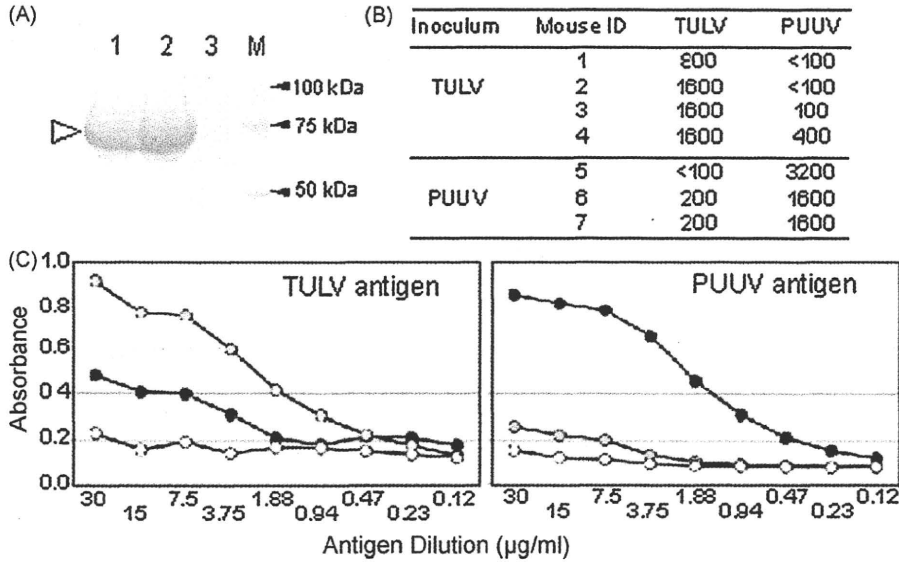


Fig. 1. (A) Antigen preparation for ELISA. The first 103 amino acids of the N-terminus of the N protein of PUUV and TULV were expressed as NUS-tagged fusion proteins in *E. coli* (BL21.DE3) by the using PET 43.1 vector and purified using a HisTrap HP column. The expected molecular weight of the truncated N-fusion proteins was 67 kDa. Lane 1, truncated N of TULV; lane 2, truncated N of PUUV; lane 3, a fraction from mock-transformed *E. coli*; M, molecular weight markers. The white arrow indicates the 67-kDa protein. (B) IFA antibody titers of sera from mice inoculated with TULV or PUUV. Sera were collected at 35 days post-inoculation. IFA antibody titers were determined as described in Materials and methods. (C) Determination of optimal antigen dilution in ELISA. The results indicate that the optimal antigen dilution for ELISA was 10 µg/ml. Gray circle, TULV-inoculated mouse serum ID4; filled circle, PUUV-inoculated mouse serum ID5; open circle, non-inoculated mouse serum.

3. Results

3.1. Development of ELISA using recombinant antigens of PUUV and TULV

Truncated N antigens of PUUV and TULV were expressed in *E. coli* and purified (Fig. 1A). The molecular weights of both recombinant antigens (truncated N tagged with the NUS protein consisting of 495 aa) were estimated to be around 70 kDa by SDS-PAGE analysis, as expected. Based on the reactivity patterns of immune sera shown as in Fig. 1C, 10 µg/ml was used in this ELISA as the antigen

concentration about twofold higher than the minimal amount that gave plateau absorbance values.

3.2. Antibody responses in experimentally inoculated laboratory-bred mice and Japanese grass voles

IFA antibody titers of immune mouse sera to TULV and PUUV were measured (Fig. 1B). All sera showed significantly high antibody titers against homologous antigens, while exhibiting lower cross-reactivities with heterologous antigens. Immune sera from mouse ID 4 and ID 5 were used as standard sera at 1:200 dilution to titrate ELISA

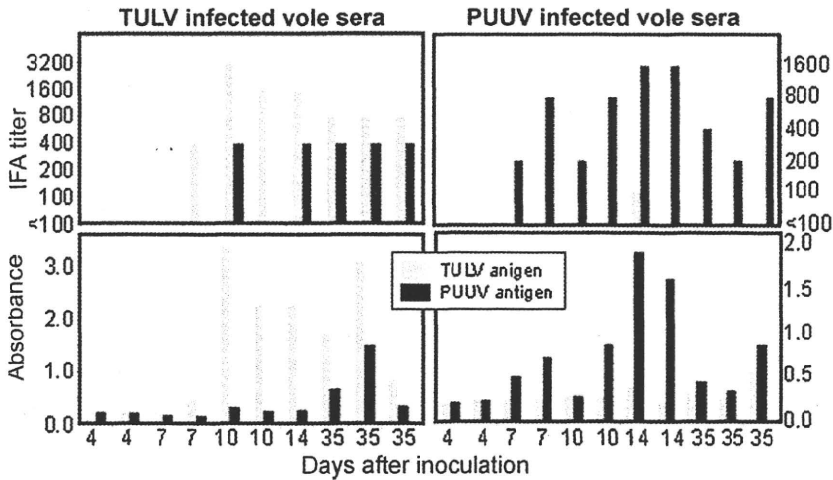


Fig. 2. IFA antibody titers of sera from Japanese grass voles (*Microtus montebelli*) inoculated with TULV and PUUV (upper panels). ELISA absorbance readings to the truncated N antigens are shown at 1:200 dilution (lower panels). Gray bars, IFA titers or ELISA absorbance readings against the TULV antigen; black bars, those against the PUUV antigen.

antigens of TULV and PUUV, respectively (Fig. 1C). Similar to the results of IFA tests, higher absorbance values were observed for homologous combinations. The antibody response in Japanese grass voles inoculated with TULV or PUUV was examined by IFA and ELISA (Fig. 2). The antibodies were first detected at around 7 days post-inoculation in both TULV- and PUUV-inoculated voles. Thereafter, significantly higher antibody titers were obtained in sera from both TULV- and PUUV-infected voles to homologous viruses by both IFA and ELISA. Cross-reactivities with heterologous antigen and antisera combinations were lower in both tests except for the IFA test using TULV-infected vole sera. TULV-infected vole sera showed 1/8 to 1/2 lower cross-reactivities to PUUV antigen in IFA in 7 voles. PUUV-infected vole sera showed no cross-reactivity to TULV antigen by IFA except for one serum. In ELISA, higher reactivities with homologous combinations were observed in both TULV- and PUUV-inoculated vole sera (Fig. 2, lower panels).

3.3. Reactivity of sera from patients with HFRS caused by PUUV

Sera from patients with HFRS in Germany were used to evaluate the serological cross-reactivity with TULV using ELISA [16]. Although most of the sera exhibited higher ELISA absorbance values with the PUUV antigen, the cross-reactivity with TULV was also high (Fig. 3).

3.4. Reactivity of vole sera naturally infected with PUUV

The serological cross-reactivity between PUUV and TULV was examined using vole sera that were naturally infected with PUUV. As shown in Fig. 4, three of the vole sera (#13, 49, 73) showed higher absorbance values with PUUV in the ELISA, but nearly 50% cross-reactivity with TULV was also observed. Meanwhile, the cross-reactivity

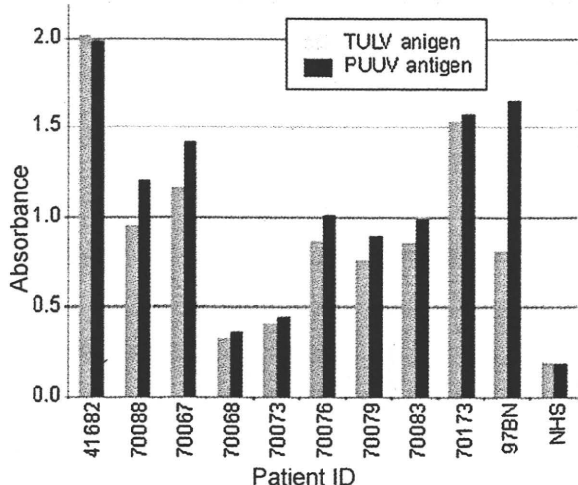


Fig. 3. Cross-reactivities of PUUV in sera from HFRS patients to the TULV and PUUV antigens by ELISA. HFRS patient sera from Germany were confirmed to be infected with PUUV by FRNT assay [16]. Gray bars, ELISA readings against the TULV antigen; black bars, those against the PUUV antigen.

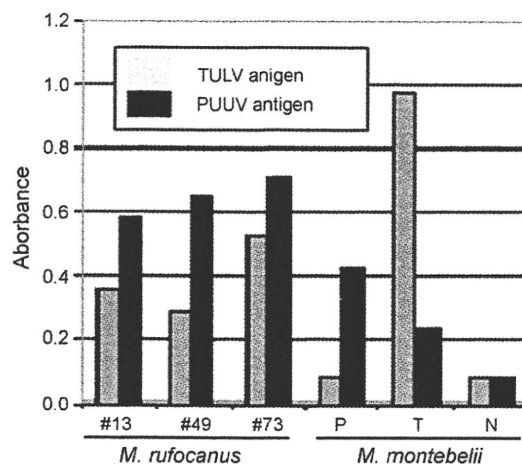


Fig. 4. Cross-reactivities of sera from *Myodes rufocanus* from Hokkaido, Japan against the TULV and PUUV antigens. Wild-captured *M. rufocanus* voles were confirmed to be infected with HOKV. Sera from experimentally inoculated Japanese grass voles were used as positive controls and a non-inoculated vole serum was used as a negative control serum. Gray bars, ELISA readings against the TULV antigen; black bars, those against the PUUV antigen.

Table 2
Viral genome detection from lung tissue by RT-PCR.

Days after inoculation	TULV-inoculated	PUUV-inoculated
4	++	–
4	–	–
7	+	–
7	++	–
10	–	–
10	–	–
14	–	–
14	ND	–
35	–	–
35	–	–
35	–	–
Positive control ^a	++	++

^a RT-PCR amplification from 10,000 Vero E6 cells inoculated with PUUV or TULV.

with heterologous antigens was lower in vole sera experimentally inoculated with TULV or PUUV (Fig. 4, P and T).

3.5. RT-PCR from lung tissues

Detection of the hantavirus genome from lung tissues of experimentally inoculated voles was carried out by RT-PCR. As shown in Table 2, the voles at 4 and 7 days after TULV inoculation were positive by RT-PCR, while no virus genome was detected thereafter. No virus genome was observed in any of the voles infected with PUUV. No virus genome was detected in lung tissues from mice at 35 days after inoculation with TULV or PUUV (data not shown).

4. Discussion

We successfully established an ELISA system using the N-terminal 103 aa of the truncated N protein of TULV and

PUUV expressed in *E. coli*. Mouse sera experimentally infected with TULV and PUUV were utilized to adjust the amounts of both antigens equally from their efficacy of antibody binding. Using this ELISA, the antigenic properties of TULV and PUUV were examined by comparing the ELISA reactivity of sera from experimentally infected laboratory-bred Japanese grass voles (*M. montebelli*), mice inoculated with TULV and PUUV, human patients with PUUV, and naturally PUUV-infected voles. The ELISA with the truncated TULV and PUUV N antigens could detect the antibody effectively. The reactivity of TULV and PUUV N with homologous combinations was stronger than that of the heterologous ones in the experimentally infected vole sera. In sera from wild *M. rufocanus* infected with Japanese PUUV, the TULV antigen could serologically differentiate about 50% of the cross-reactivities as compared to the homologous ones. However, in the PUUV patient sera, the TULV antigen cross-reacted strongly and nearly equal absorbance values were obtained for both antigens.

TULV recombinant N proteins expressed by baculovirus, yeast, and *E. coli* have been developed and utilized successfully as antigens for ELISA [15]. Studies have shown that *E. coli*-expressed N-terminal TULV (1–61 aa) and PUUV (1–79 aa) antigens were highly antigenic and contained at least four epitopes, two of which are conserved in all hantaviruses carried by rodents within the subfamily Arvicolinae [15]. The TULV (1–61 aa) antigen also showed lower cross-reactivity with sera obtained from naturally PUUV-infected voles. The results of the current study are in agreement with the results of a previous study, confirming that the N-terminal region is immunogenic and a useful antigen for ELISA. However, sera from voles experimentally inoculated with TULV showed 1/4 lower reactivity with homologous TULV N (1–61 aa) than with heterologous PUUV N (1–79 aa). In our study, TULV and PUUV N (1–103 aa) showed higher reactivity in the homologous combination in sera from experimentally infected voles. Recently, the structure of the N-terminal region of hantavirus N protein has been reported, and it has been shown that N-terminal 74 aa are needed for the coiled-coil structure of the inner N protein [30]. In addition, at least three epitopes, aa 17–59, 66–78, and 79–91, were found in the N-terminal region of the N protein [26]. According to these observations, the truncated N protein of aa 1–61 might lose the stable coiled-coil structure and several epitopes. Therefore, the role of the N-terminal region between aa 62 and 103 might be related to the antigenic efficacy of the truncated N and, consequently, the homologous reactivity is lowered. Instead, the first 103 aa of the truncated N protein of TULV and PUUV used in this study could differentiate homologous and heterologous infections in rodent sera. However, the reason for the inability to differentiate PUUV infection and TULV infection in human sera is unclear.

A laboratory-bred Japanese grass vole (*M. montebelli*) was used to evaluate its potential use as a model animal for *Microtus*-borne hantaviruses. Since it produces high titers of antibody to the inoculated virus, the Japanese grass vole is considered to be susceptible to both TULV and PUUV infection. The virus genome was detected from the organs of TULV-infected Japanese grass voles in the early phase of

infection, but not from PUUV-infected voles. Therefore, pathogenesis in this vole was different from that in natural hosts of TULV and PUUV.

Due to low susceptibility, laboratory mice are not usually used for PUUV experiments. Instead, Mongolian gerbils have been used for preparing the PUUV antigen [14,20]. Klingstrom et al., however, reported that laboratory-bred BALB/c mice displayed susceptibility to PUUV and Topografov virus (TOPV) [10], but, unexpectedly, mice were also found to be susceptible to PUUV strain CG1820 and TULV strain Moravia in this study. Therefore, PUUV susceptibility in laboratory mice must be examined carefully using various strains of PUUV.

In this study, we evaluated ELISA with N-terminal recombinant N protein of TULV and PUUV by using immune sera prepared in laboratory-bred mice and voles. The ELISA showed that cross-reactivity between antisera against TULV and PUUV in rodent sera was lower than heterologous antigen. Therefore, the use of both antigens of TULV and PUUV for serological tests is recommended for epizootiological studies on captured wild voles. Since PUUV patient sera cross-reacted strongly to TULV antigen, serotyping by neutralization tests would be required so as not to overlook TULV infection cases, particularly in an area where both viruses are co-circulating.

Acknowledgments

This study was supported in part by a grant from the Global COE Program, "Establishment of International Collaboration Center for Zoonosis Control" and Grants-in-Aid for Scientific Research and the Development of Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. This study was financially supported by a Grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Welfare, and Labor of Japan. We would like to thank Dr. Rainer Ulrich, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute for Novel and Emerging Infectious Diseases, D-17493 Greifswald–Insel Riems, Germany, for providing HFRS patient sera. We would also like to acknowledge text check for grammatical revisions in the final draft.

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Truncated Hantavirus Nucleocapsid Proteins for Serotyping Sin Nombre, Andes, and Laguna Negra Hantavirus Infections in Humans and Rodents[∇]

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Received 13 January 2010/Returned for modification 8 February 2010/Accepted 12 March 2010

Sin Nombre virus (SNV), Andes virus (ANDV), and Laguna Negra virus (LANV) have been known as the dominant causative agents of hantavirus pulmonary syndrome (HPS). ANDV and LANV, with different patterns of pathogenicity, exist in a sympatric relationship. Moreover, there is documented evidence of person-to-person transmission of ANDV. Therefore, it is important in clinical medicine and epidemiology to know the serotype of a hantavirus causing infection. Truncated SNV, ANDV, and LANV recombinant nucleocapsid proteins (trNs) missing 99 N-terminal amino acids (trN100) were expressed using a baculovirus system, and their applicability for serotyping SNV, ANDV, and LANV infection by the use of enzyme-linked immunosorbent assays (ELISA) was examined. HPS patient sera and natural-reservoir rodent sera infected with SNV, ANDV, and LANV showed the highest optical density (OD) values for homologous trN100 antigens. Since even patient sera with lower IgM and IgG antibody titers were serotyped, the trN100s are therefore considered useful for serotyping with early-acute-phase sera. In contrast, assays testing whole recombinant nucleocapsid protein antigens of SNV, ANDV, and LANV expressed in *Escherichia coli* detected homologous and heterologous antibodies equally. These results indicated that a screening ELISA using an *E. coli*-expressed antigen followed by a serotyping ELISA using trN100s is useful for epidemiological surveillance in regions where two or more hantavirus species cocirculate.

Hantaviruses belong to the *Hantavirus* genus in the *Bunyaviridae* family. Hantaviruses cause two rodent-borne febrile illnesses in humans, hemorrhagic fever with renal syndrome (HFRS) in the Old World and hantavirus pulmonary syndrome (HPS) in the New World (11, 25). So far, 23 virus species have been registered within the *Hantavirus* genus. Among the Old World hantaviruses, Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava-Belgrade virus (DOBV), and Puumala virus (PUUV) are commonly associated with HFRS, while the New World species Sin Nombre virus (SNV), New York virus (NYV), Black Creek Canal virus (BCCV), Andes virus (ANDV), and Laguna Negra virus (LANV) regularly cause HPS in the New World (25).

Since 1993, when HPS was first identified in the New World (20), many new hantaviruses with or without human disease have been described throughout North, Central, and South America. ANDV and LANV, with different pathogenicity patterns and with approximately 40% and 15% mortality rates,

respectively, exist in a sympatric relationship in Argentina (10, 14). Moreover, there is documented evidence of person-to-person transmission of some kind of ANDV strain (15, 22). However, since the neutralization test (NT), which is the only serological assay available for serotyping, needs specialized techniques and equipment and requires a containment laboratory for virus manipulation (2), serological typing of ANDV and LANV infection has been limited.

Hantavirus virions contain three segmented negative-sense RNAs designated S, M, and L: they encode a nucleocapsid protein (N), enveloped glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase (L protein), respectively (4). Hantavirus N is the most abundant viral component in both virions and infected cells and can form a stable trimer (7, 12). The N of Old World hantaviruses possesses immunodominant linear epitopes around the first 100 amino acids (aa) of the N terminus (6, 8, 32). These N-terminal epitopes cross-reacted with all of the Old World hantaviruses except PUUV. On the other hand, the variable region at around 230 to 302 aa forms serotype-specific epitopes after multimerization of N (30, 36).

We have developed a baculovirus that expresses truncated recombinant N (trN) lacking 49 aa of the N-terminal end of the N (trN50). trN50 showed decreased reactivity to cross-reactive antibodies but preserved reactivity to serotype-specific anti-

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[∇] Published ahead of print on 24 March 2010.

bodies after multimerization of trNs. Use of an enzyme-linked immunosorbent assay (ELISA) system with trN50 successfully differentiated four hantavirus infections with HTNV, SEOV, DOBV, and Thailand virus (THAIV) in HFRS patient and rodent sera. Therefore, it seemed that the ELISA was a faster, safer, and simpler serotyping method than and an effective substitute for the NT (2, 19).

In the present study, we attempted to apply similar N-terminal deletion N antigens for serotyping using ELISA. We first selected SNV, ANDV, and LANV, 3 New World hantaviruses that are major causative agents of HPS, and examined the multimerization of trNs and their antigenic efficacy. We then used the trNs for serotyping of SNV, ANDV, and LANV infections.

MATERIALS AND METHODS

cDNAs and cells. cDNAs containing coding information for N of SNV strain SN 77734 (5), ANDV (23), and LANV strain 510B (9) were used. High Five cells (Invitrogen, Groningen, Netherlands) were grown in Grace's insect cell culture medium (Invitrogen) supplemented with 10% fetal bovine serum as previously described (2).

MAbs and human and rodent sera. Monoclonal antibodies (MAbs) to N of HTNV and PUUV were used for antigenic characterization of N by indirect immunofluorescence assay (IFA). MAbs 2E12, 4C3, 4E5, GBO4, ECO2, 1C12, and ECO1 recognize the N-terminal epitope of N. MAbs E5/G6 and F23A1 recognize aa 165 to 173 and aa 291 to 402 of N, respectively. The epitope for MAb C16D11 is unknown (21, 24, 34).

Eleven serum samples from HPS patients infected with SNV in the United States were kindly supplied by Brian Hjelle of the University of New Mexico Health Sciences Center. Eleven serum samples from HPS patients infected with ANDV and six serum samples from HPS patients infected with LANV were obtained from Argentina. Thirty-one serum samples from *Peromyscus maniculatus* infected with SNV and five hantavirus-negative serum samples from *Peromyscus maniculatus* were obtained from Canada. Twenty-three serum samples from Sigmodontinae rodents (*Oligoryzomys longicaudatus*, *Oligoryzomys flavescens*, and *Akodon azarue*) infected with ANDV and live serum samples from LANV-infected *Calomys callosus* were obtained from Argentina. Hantavirus-negative human sera were obtained from healthy volunteers. Negative-control rodent sera (*Sigmodon hispidus*) were kindly supplied by Kimiyuki Tsuchiya of Applied Biology Co. Ltd., Tokyo, Japan. The types of virus in sera from the patients and rodents were determined by detection of the virus genome by reverse transcription-PCR (RT-PCR).

Construction of recombinant baculoviruses expressing whole rNs and trNs. The gene encoding whole N (aa 1 to 428) and truncated genes encoding truncated recombinant N (aa 50 to 428 [trN50] and aa 100 to 428 [trN100]) were amplified from cDNA of the S segment by the use of PCR. The primers listed below amplified whole and truncated S segments. A 5' Spel site and a 3' XhoI site were introduced into the primers (both sites are shown in italics below). The sequences of the primers (forward and reverse, respectively) were as follows (underlining indicates an added start codon): for SNV whole rN, 5'-gacactagrat gaccaccctcaagaa-3' and 5'-tacctcgattaaagttaagttaagtggttc-3'; for ANDV whole rN, 5'-aaaactagratgagcaacctccaagaa-3' and 5'-ttactcgattacagctttaagggc-3'; for LANV whole rN, 5'-taactagratgagcaacctccaagaa-3' and 5'-actctcgattagagtttaggggttc-3'; for SNV trN50, 5'-tcgactagratggctgtctgctgaggg-3' and 5'-tacctcg atttaagttaagttaagtggttc-3'; for ANDV trN50, 5'-agaactagratggctgtctgctgaggg-3' and 5'-ttactcgattacagctttaagggc-3'; for LANV trN50, 5'-agcactagratggctgtctgctgaggg-3' and 5'-actctcgattagagtttaggggttc-3'; for SNV trN100, 5'-tcgactagratggctgtctgctgaggg-3' and 5'-tacctcgattaaagttaagttaagtggttc-3'; for ANDV trN100, 5'-cgaactagratgagctgctgaggg-3' and 5'-ttactcgattacagctttaagggc-3'; and for LANV trN100, 5'-ctgactagratgagctgctgaggg-3' and 5'-actctcgattag agtttaggggttc-3'. After amplification, the DNA fractions were subcloned into pFastBac1 (Invitrogen) and were expressed using a Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions. Mock baculovirus was made from pFastBac1. The titers of recombinant baculoviruses in the culture supernatant were determined by calculation of 50% tissue culture infective dose (TCID₅₀) values with High Five cells.

Preparation of whole rNs and trNs expressed by baculoviruses. High Five cells were infected for 3 days with recombinant baculoviruses at a multiplicity of infection (MOI) of 1. Collection and lysis of infected cells were performed using

previously published methods (2). Briefly, infected High Five cells were collected in phosphate-buffered saline (PBS) with 2.5×10^6 cells/ml and sonicated. The cell lysate containing recombinant Ns (rNs) was used as an IgG and IgM ELISA antigen. The cell lysate infected with mock baculovirus was used as a negative control. Expression of rNs of SNV, ANDV, and LANV was confirmed by Western blotting (WB) (data not shown) using previously published methods (35). High Five cells expressing whole rNs of PUUV and HTNV were prepared as previously described (2). High Five cells expressing whole rNs and trNs were used for IFA.

Preparation of rNs expressed by *E. coli*. The whole rNs of SNV, ANDV, and LANV were expressed in *Escherichia coli*. DNA fractions containing the entire coding region of N were made by digestion of pFastBac1 with Sall and XhoI. The DNA fractions were subcloned into the pET43b vector (Merck KGaA, Darmstadt, Germany) and transfected into *E. coli* strain BL21(DE3) (Merck KGaA). A single colony was inoculated into Circle growth medium (MP Biomedicals, Morgan Irvine, CA) containing ampicillin (50 µg/ml) for small-scale culture incubation at 37°C overnight. The culture fluid was then centrifuged, the collected cells were inoculated into 100 ml of Circle growth medium, and isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction was performed according to the procedure for pET system expression.

The cultured cells were collected by centrifugation, resuspended in 5 ml of 0.5 M NaCl binding buffer (0.5 M NaCl, 20 mM imidazole, 20 mM potassium phosphate), and sonicated on ice four times for 15 s each time. Thereafter, the fusion protein was purified using a HisTrap HP column (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. A negative antigen that included the Nus-tag protein, made from the pET43b vector but not including the hantavirus gene, was used as a negative control. The first 103 aa of the N-terminal region of HTNV N (HTNV HS103) were prepared as previously described (34). The purity of the recombinant proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown).

IFA. To characterize the rNs expressed by the baculovirus, we used a previously described IFA (18, 33). Acetone-fixed smears of High Five cells infected with recombinant baculoviruses were used as antigens.

Detection of multimerized rNs. To detect multimerization of the rNs expressed by the baculovirus, competitive-sandwich ELISA was performed with MAb E5/G6 recognizing aa 165 to 173 as a capture antibody. Briefly, rNs were captured on the plate with MAb E5/G6 followed by detection with the same MAb E5/G6. A positive reaction with this ELISA indicates that the antigens are forming a multimer (36).

ELISA with whole rNs expressed by *E. coli*. By the use of whole rNs expressed by *E. coli*, 96-well plates were first coated overnight at 4°C with 1 µg/ml of whole rNs in PBS as a capture antigen. After being washed three times with PBS containing 0.05% Tween 20 (PBS-T), the plates were then blocked with PBS containing 3% bovine serum albumin (BSA) for 1 h at 37°C. After blocking, patient and rodent sera were diluted 1:200 with ELISA buffer (PBS containing 0.5% BSA and 0.05% Tween 20) and added to the plates for 1 h at 37°C. After being washed with PBS-T, bound antibody was detected with peroxidase-labeled goat anti-human IgG (H+L) antibody (KPL, Gaithersburg, MD) for patient sera, horseradish peroxidase (HRP)-labeled goat anti-*Peromyscus leucopus* IgG (H+L) antibody (KPL) for *Peromyscus* rodent sera, or biotin-labeled mouse anti-*Sigmodon hispidus* IgG antibody for Sigmodontinae rodent sera for 1 h at 37°C. As another step using biotin-labeled mouse anti-*Sigmodon hispidus* IgG antibody, streptavidin-HRP conjugate (Prozyme, San Leandro, CA) was subjected to reactions for 30 min at 37°C. After being washed, color reactions were performed with *o*-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St. Louis, MO) and allowed to develop for 10 to 15 min. Absorbance was measured at 450 nm by using a SpectraMax 340 microplate spectrophotometer (Molecular Device, Sunnyvale, CA). HTNV HS103 antigen was used as a control for the Old World hantavirus experiments for HTNV infections only. HTNV HS103 antigen has 103 aa of the N terminus and includes cross-reactive epitopes.

Serotyping ELISA with trNs expressed by baculovirus. The serotyping ELISA was carried out as previously described (2, 19). The plates were coated overnight at 4°C with MAb E5/G6 (2 µg/ml in PBS) as a capture antibody. Washing and blocking were carried out in the same manner. After three washes, 10-fold dilutions of rNs were added to the plates for 1 h at 37°C. After washing with PBS-T as described above, 200-fold dilutions of patient and rodent sera were added to the plates and incubated for 1 h at 37°C. The secondary antibody and color development results were the same as those for the ELISA with whole rNs.

IgM ELISA. The IgM ELISA was carried out as previously described (17). The plates were coated overnight at 4°C with goat anti-human µ-chain antibody (Cappel, Aurora, OH) in 100 mM carbonate buffer as a capture antibody.

TABLE 1. Antigenic characterization of rNs expressed by recombinant baculovirus with MAb in the IFA test^a

Origin	MAb	Epitope	IFA result ^b											
			Whole rN						trN50 (New World hantavirus)			trN100 (New World hantavirus)		
			Old World hantavirus			New World hantavirus			SNV	ANDV	LANV	SNV	ANDV	LANV
PUUV	HTNV	SEOV	SNV	ANDV	LANV	SNV	ANDV	LANV	SNV	ANDV	LANV			
PUUV	2E12	N terminus	+	±	±	+	+	+	-	-	-	-	-	-
	4C3	N terminus	+	+	+	+	+	+	-	-	-	-	-	-
	4E5	N terminus	+	+	±	+	+	+	-	-	-	-	-	-
	GBO4 ^c	N terminus	+	+	+	+	+	+	±	+	±	-	-	-
HTNV	ECO2	aa 1-33	-	+	+	-	-	-	-	-	-	-	-	-
	1C12	N terminus	+	+	+	+	+	+	-	-	-	-	-	-
	ECO1 ^c	aa 34-103	+	+	+	+	+	+	-	-	-	-	-	-
	C16D11	Unknown	+	+	+	-	+	+	-	+	+	-	-	-
	E5/G6	aa 165-173	+	+	+	+	+	+	+	+	+	+	+	+
	F23A1	aa 291-402	-	+	+	+	+	+	+	+	+	+	+	+

^a PUUV, Puumala virus; HTNV, Hantaan virus; SEOV, Seoul virus.

^b Symbols: +, positive IFA result of >1:1 (1:100 for ascitic fluid samples); ±, scarcely positive IFA result; -, negative IFA result.

^c The sample was ascitic fluid.

Washing and blocking were carried out in the same manner. After three washes with PBS-T, 200-fold dilutions of patient sera were added to the plates and incubated for 1 h at 37°C. After washing with PBS-T as described above, 10-fold dilutions of SNV whole rN for baculovirus were added to the plates for 1 h at 37°C. After washes were performed, biotin-labeled E5/G6 MAb was subjected to reactions for 1 h at 37°C. After reaction with streptavidin-HRP conjugate (Prozyme), the color was developed with TMB (3,3',5,5'-tetramethylbenzidine) for 15 min. and development was stopped with 0.5 M sulfuric acid. Absorbance was measured at 450 nm by using a SpectraMax 340 microplate spectrophotometer (Molecular Device).

RESULTS

Antigenic characterization of rNs expressed by baculovirus.

The IFA tests for antigenic profiling of whole rNs, trN50s, and trN100s expressed in High Five cells were carried out using hantavirus-specific MAbs (Table 1). Whole rNs of New World hantavirus reacted to cross-reactive MAbs (2E12, 4C3, 4E5, GBO4, 1C12, and ECO1) that recognized immunodominant epitopes of the N terminus of N and to cross-reactive MAbs (E5/G6 and F23A1) that recognized aa 165 to 173 and aa 291 to 402 of N, respectively. TrN50s of SNV, ANDV, and LANV mainly reacted with cross-reactive MAbs (E5/G6 and F23A1) but still remained cross-reactive to MAbs (GBO4 and C16D11) recognizing the N-terminal epitopes. In contrast, trN100s of SNV, ANDV, and LANV reacted to only two cross-reactive MAbs, E5/G6 and F23A1.

Detection of multimerization of rNs. Since E5/G6 reacted to rNs in the IFA, E5/G6 was used in competitive-sandwich ELISA. As shown in Fig. 1, there was no reaction to trN50s and whole rNs. This implied that the trN50s and whole rNs captured by E5/G6 could not react with E5/G6 as a detector due to competition. Thus, trN50s and whole rNs of SNV, ANDV, and LANV were found as monomers. On the other hand, there were strong reactions to trN100s, indicating that trN100s of SNV, ANDV, and LANV existed as multimers. As the serotype-specific epitopes were considered to be formed after multimerization of trNs, we selected trN100s as ELISA antigens for serotyping ELISA.

Reactivities of whole rNs and trN100s with infected sera. To examine the applicability of the recombinant antigens in

ELISA, the ELISA optical density (OD) values determined with whole rNs and trN100s in 28 human and 59 rodent serum samples were compared. As shown in Fig. 2, when tested using whole rN antigens, SNV, ANDV, and LANV infections were not differentiated because of strong cross-reaction. However, HTNV-infected sera reacted strongly only to HTNV antigen of the Old World hantavirus.

On the other hand, the trN100s of SNV, ANDV, and LANV showed serotype-specific reaction patterns. The ELISA ODs of heterologous antigens were less than half of those of homologous reactions for most of the sera tested (Fig. 3). The mean reactive rate of OD values of serum specimens for heterologous antigens was 37.9%, with 22.9% as the standard deviation (SD) of those for homologous antigens. Therefore, as the mean of the reactive rate for homologous and heterologous antigens plus twice the SD of the OD values was 83.7%, the cutoff value to distinguish homologous and heterologous reactions was tentatively determined to be 80% (Fig. 4). All of the 28 patient sera and 54 of the 59 rodent sera were serotyped. Although 5 of the rodent sera showed stronger cross-reactivity,

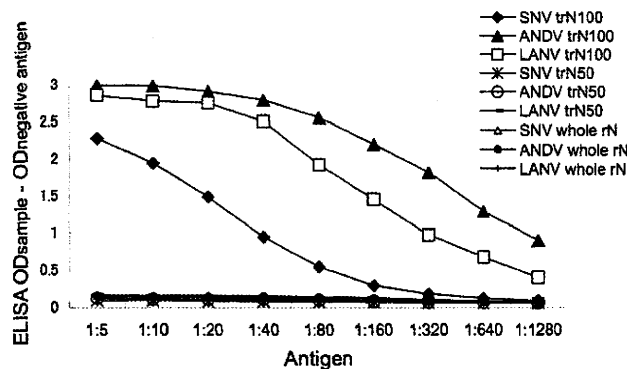


FIG. 1. Multimerization of rNs. Antigens were captured and detected with MAb E5/G6. Each antigen was diluted 1:5 to 1:1,280 and subjected to capture ELISA. The OD value was corrected by the negative control. The ELISA was performed three times in duplicate, and the means of the OD values were plotted.

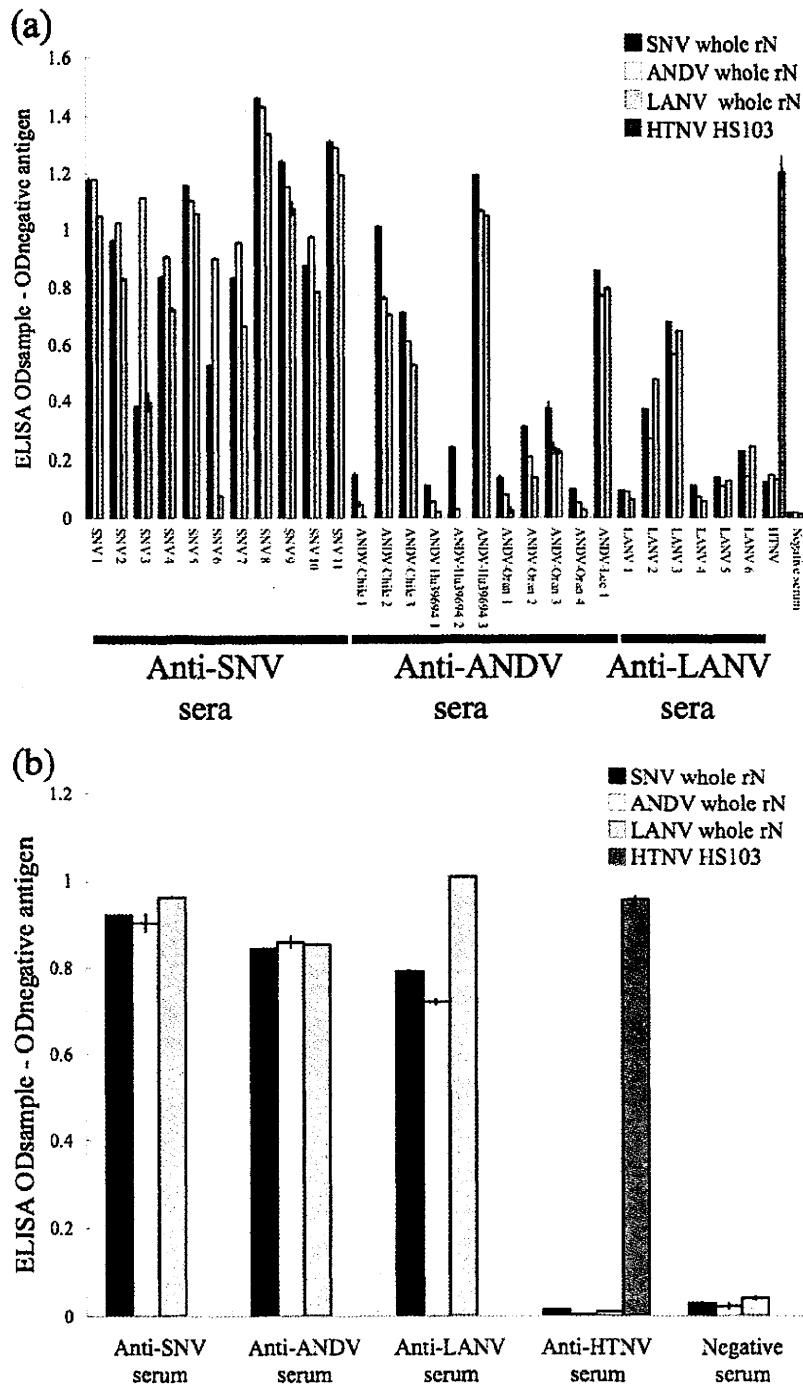


FIG. 2. (a) Reaction of whole rNs with hantavirus-positive human sera. (b) Reaction of whole rNs with representative hantavirus-positive rodent sera (anti-SNV serum, *Peromyscus maniculatus*; anti-ANDV serum, *Oligoryzomys longicaudatus*; anti-LANV serum, *Calomys callosus*; anti-HTNV serum, *Ratus norvegicus*). The total number of hantavirus-positive rodent sera was 59. HTNV-infected patient or rat sera were used as the control for Old World hantavirus infection. The OD value was corrected using the negative-control value. The ELISA was performed three times in duplicate, and the bars show the mean values.

the OD values were higher for homologous antigens, except for that of 1 *Peromyscus maniculatus* anti-SNV serum sample.

Detection of IgG and IgM antibodies to whole rN in patient sera. To investigate phase when HPS patient sera were obtained, IgG and IgM antibodies in HPS patient sera were

examined by using whole rN of SNV. As shown in Fig. 5a, they were roughly divided into 4 clusters (clusters I, II, III, and IV) based on the levels of IgG and IgM antibodies: low IgG and low IgM, low IgG and moderate IgM, high IgG and high IgM, and high IgG and low IgM. The results shown in

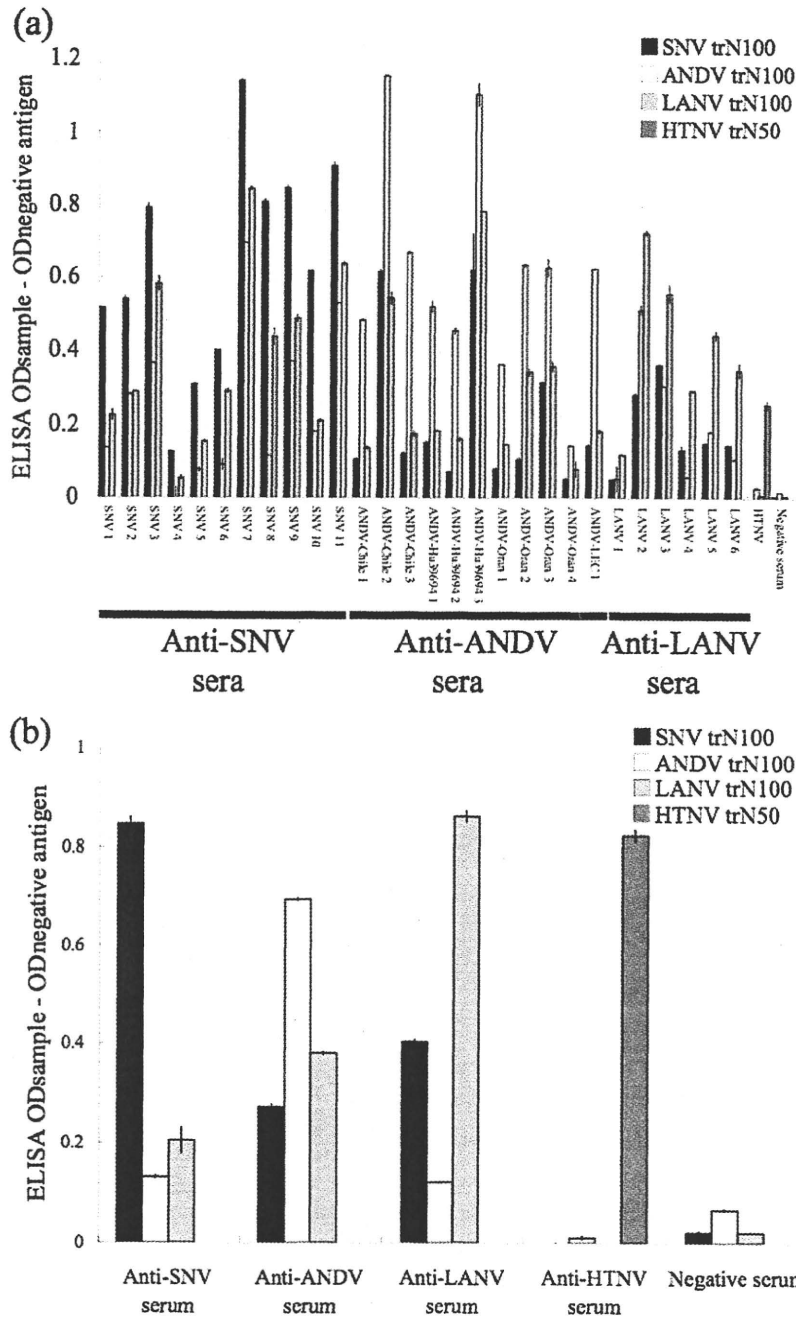


FIG. 3. (a) Reaction of trN100s with hantavirus-positive human sera. (b) Reaction patterns of trN100s with representative hantavirus-positive rodent sera (the serum samples represented in Fig. 3b were the same as those in Fig. 2b) in ELISA. A total of 28 patient serum samples and 59 rodent serum samples were used to assess our serotyping ELISA system. HTNV-infected patient or rat sera were used as the control for Old World hantavirus infection. The OD value was corrected using the negative-control value. The ELISA was performed three times in duplicate, and the bars show the mean values.

Fig. 3a were rearranged in the order of sera classified into each cluster (Fig. 5b). As shown, irrespective of titers of the IgM and IgG antibody, they were serotyped.

DISCUSSION

At present, NT is the only method for differentiating serotypes of New World hantavirus infections (31). In this study,

we showed that ELISA using N-terminally truncated trN100 antigens of three HPS-associated hantavirus species (SNV, ANDV, and LANV) was able to serologically differentiate serum specimens from SNV-, ANDV-, and LANV-infected patients or rodents (Fig. 3). On the other hand, whole rN antigens showed strong cross-reactivity with heterologous sera (Fig. 2). Therefore, screening by ELISA using whole rNs followed by serotyping using trN100s can be recommended as a

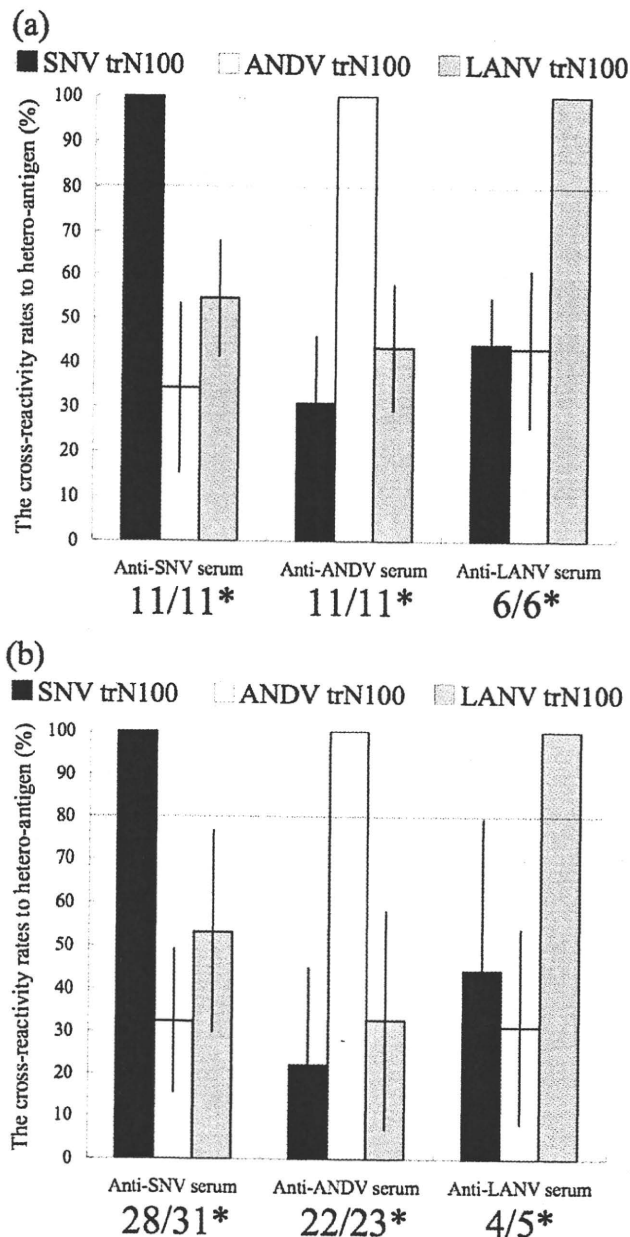


FIG. 4. The mean OD values for rates of reaction to heterologous antigens (y axis). (a) Patterns of cross-reactions of hantavirus-positive human sera to heteroantigens. (b) Patterns of cross-reactions of hantavirus-positive rodent sera to heteroantigens. The mean OD value for rates of reaction of serum specimens to heterologous antigens was 37.9%, with an SD of 22.9% for those to homologous antigens. Therefore, as the mean of the rates of reaction for homologous and heterologous antigens plus twice the SD of the OD values was 83.7%, the cutoff value to distinguish homologous and heterologous reactions was tentatively determined to be 80%. The dotted lines indicate the cutoff value to identify the serotype. *, the numbers indicate the number of serotyped samples (left) and the total number of samples (right).

rapid and practical system for hantavirus seroepidemiology and diagnosis.

To date, MAbs to N of New World hantaviruses have not been readily available (30). Consequently, antigenic character-

ization of rNs was indirectly confirmed using MAbs to Old World hantavirus by IFA. As we expected from our previous study (34), most of the MAbs that recognized immunodominant epitopes of the N terminus of N for Old World hantaviruses reacted to whole rNs of SNV, ANDV, and LANV but not to trN100s (Table 1). These results indicate that the first 100 aa of the N terminus of N possess immunodominant, cross-reactive epitopes, as previously reported (6, 8, 32). Therefore, it is thought that the structure of N of New World hantavirus is similar to that of N of Old World hantavirus.

It has been shown that the Old World hantavirus N built whole rN-whole rN or trN50-trN50 multimers and that both multimers were able to form multimerization-dependent serotype-specific epitopes (36). In this study, the trN50s and whole rNs of New World hantavirus were found as monomeric N in sandwich ELISA (Fig. 1). In contrast, the trN100s of SNV, ANDV, and LANV were detected as multimers (Fig. 1). These results support those of previous studies indicating that the first 100 aa of the N terminus did not contribute to N-N interaction but rather were inhibitory for homotypic interaction (12, 36). Multimerization of SNV N was confirmed by a yeast two-hybrid method (1). Thus, we believe that intact Ns of SNV, ANDV, and LANV form multimers in both virions and infected cells. It was considered that such a structural error of rNs was caused by limitations of the baculovirus expression system used in this study. In spite of the uncertainties of the expression system, trN100s were useful as serotyping antigens. Interestingly, the trN50 and whole rN of BCCV were detected as multimers in E5/G6-capture E5/G6-detected ELISA (data not shown). Thus, the functional roles of the N-terminal regions of New World hantaviruses N differ with respect to the capacity to form multimers.

In human cases, it is generally possible to detect viral RNA by RT-PCR in peripheral blood mononuclear cells and in serum samples during the febrile prodrome and early in the course of the cardiopulmonary phase. However, it is difficult to detect viral RNA in blood by the use of RT-PCR after the cardiopulmonary phase (13, 16, 28). Sera in which IgG and IgM antibody titers are low can be considered to represent a prodrome phase, according to previous data (3, 26, 27, 29). On the basis of the IgM and IgG antibody titers, the patient sera were divided into 4 clusters (Fig. 5a). Since clusters I and II contain serum specimens showing low or moderate IgM titers with low IgG antibody titers, the sera might have been obtained early after the onset of infection. Nevertheless, the HPS patient sera in clusters I and II were serotyped in this study (Fig. 5b). Therefore, the trN100-based typing ELISA is useful not only for latter-phase sera but also for early-acute-phase patient sera. Early differential diagnosis might contribute to the treatment effect in the future.

In conclusion, serologic diagnosis by ELISA with trN100s is rapid, simple, and safe in comparison to NT. This method is expected to be useful for clinical diagnosis and epidemiological surveillance in regions where two or more hantaviruses cocirculate. Although the percentage of amino acid sequence identity between ANDV N and LANV N used in this study was high (90.0%), trN100s were able to reduce cross-reactivity to heterologous sera. Thus, it might be possible to apply this system to other hantaviruses.

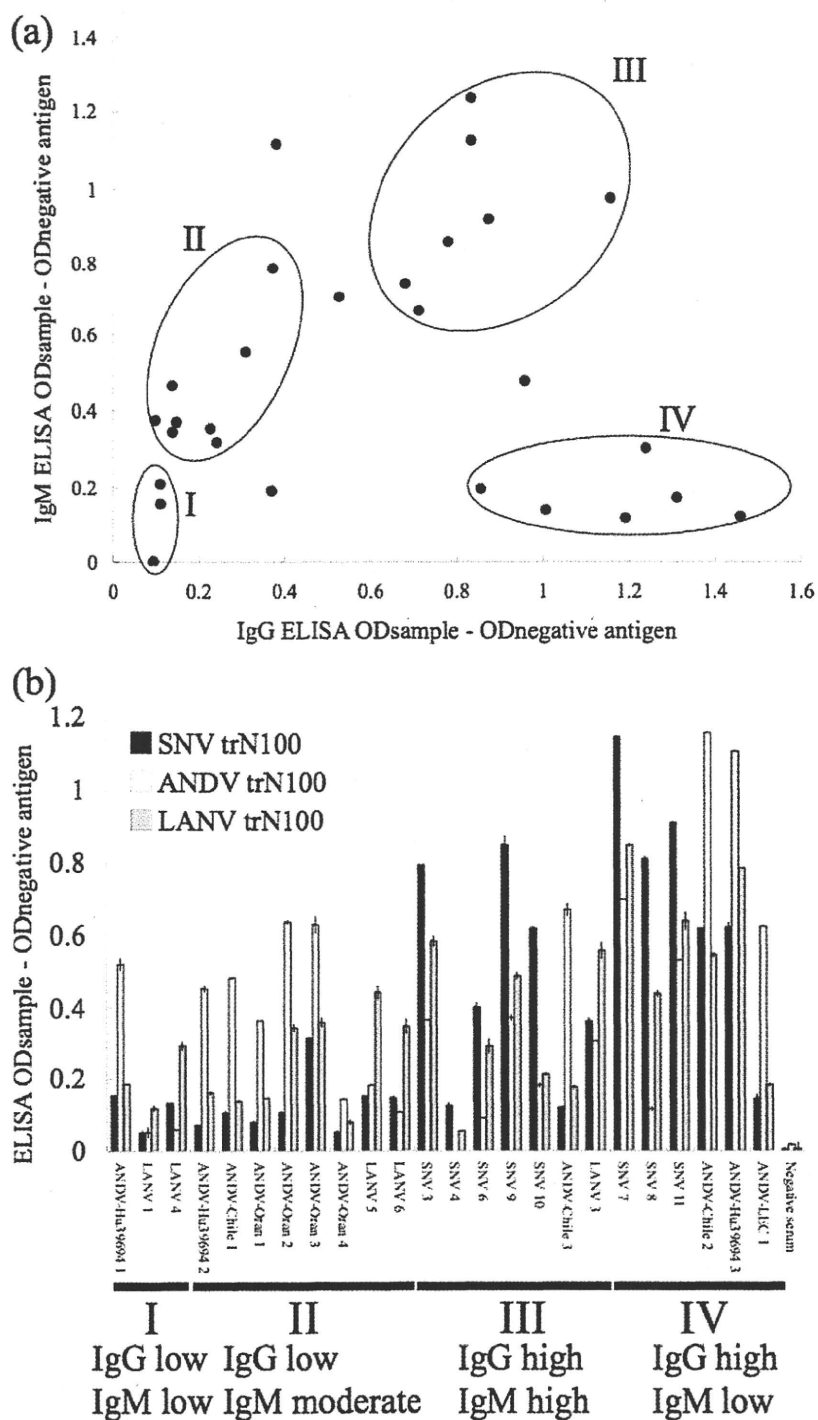


FIG. 5. (a) Patterns of IgM and IgG ELISA OD values for human cases. The sera were divided into four clusters (clusters I to IV) by the patterns of IgM and IgG. The OD value was corrected using the negative-control value. (b) Patterns of reactions of trN100s with hantavirus-positive human sera in ELISA were arranged by phase. Only ANDV-Chile 2 and ANDV-Hu39694 3 have been known to be convalescent-phase sera. The rest of the serum samples of those two patients were recorded as acute-phase samples, but details are unknown.

ACKNOWLEDGMENTS

This work was supported by the Global COE program Establishment of International Collaboration Center for Zoonosis Control from the Japanese Ministry of Education, Culture, Sports, Science and Technology. This work was also partially supported by Grants-in-Aid for Scientific Research and the Development of Scientific Research

from the Japanese Ministry of Health, Labor and Welfare (grant 200829018A). This study was supported in part by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT, Japan.

We also acknowledge Stewart Chisholm of the Stewart English School (S.E.S.) for revising the grammar in the final draft.

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effective programs to curtail the spread of TB within the country and the region; in this era of globalization, it is required for the successful control of TB worldwide.

Acknowledgments

We thank the Center de Recherche et Formation (SEREFO) team members for their support. We also thank the clinical staff, Souleymane Diallo, Sounkalo Dao, Samba Diop, Bindongo P.P. Dembele, Hamadou Kassambara, Drissa Goita, and Ousmane M'baye for their efforts in patient recruitment; and the laboratory staff, Guindo Oumar, Djeneba Dabitaou, Hama Diallo, Yeya D.S. Sarro, Mariam Tall, Nadie Coulibaly, and Bourahima Kone for patient screening and timely completion of all laboratory results. We also thank Mark Parta for help with the spoligotyping assay, Ousmane Koita for editorial help, and Christian Yoder for supporting the construction of the Biosafety Level 3 laboratory.

This work was conducted at the Mali International Centers for Excellence in Research site funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA.

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DOI: 10.201/eid1602.090501

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Hemorrhagic Fever with Renal Syndrome, Vietnam

To the Editor: Hantaviruses are primarily rodent borne and can cause hemorrhagic fever with renal syndrome (HFRS) in persons who inhale aerosolized excreta from infected rodents. The clinical characteristics of HFRS are fever, hemorrhage, and varying degrees of renal and hepatic dysfunction. Although HFRS is endemic primarily to Eurasian regions, there is serologic evidence of hantavirus infections in rodents and humans worldwide (1). Little is known about the occurrence of hantavirus infection in rodents or humans in Vietnam. One study found 5.4% prevalence of antibodies against Hantaan 76–118 and Puumala strains among residents of the Hanoi Metropolitan (2), whereas another study in southern Vietnam did not find evidence of hantavirus infection in humans (3). We describe autochthonous HFRS from Vietnam, possible reservoir hosts, and the follow-up investigation, which implies the presence of a strain of Seoul virus (SEOV).

The case-patient was a previously healthy 25-year-old nurse working in a referral hospital and residing in a semi-urban district of Ho Chi Minh City. On September 23, 2008, she was admitted to the referral hospital with a history of high fever, chills, myalgia, nausea, vomiting, hematuria, and abdominal and lower back pain for 3 days. Physical examination showed a body

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temperature of >39°C, petechiae, mild dehydration and hypotension, with otherwise unremarkable vital signs. Hematologic tests showed 13,300 leukocytes/mm³, 167,000 thrombocytes/mm³, and hematocrit of 31%. Urinalysis showed grave hematuria (3+), proteinuria (2+), and leukouria (2+).

Three days after admission, acute renal failure with relative oliguria (0.85 L/24 h) developed, as well as uremia (26.4 mg/dL), creatinemia (0.98 mg/dL), and abnormal liver function (aspartate aminotransferase 49 U/L and alanine transferase 60 U/L). The following day the patient had dyspnea and became agitated. Ultrasound examination showed pleural effusion, parietal pericardial effusion, peritoneal ascites, hepatomegaly, and renal thickening. Six days after admission, diuretic problems developed in the patient (3.7 L/24 h), her dyspnea resolved, and she became afebrile. Ten days after admission, the patient's hematuria resolved, and renal and liver functions gradually recovered; she was discharged after 29 days of hospitalization.

Immunoglobulin (Ig) M and IgG against Hantaan recombinant nucleocapsid protein antigen were detected in the case-patient's acute-phase and convalescent-phase serum samples, respectively, by ELISA (4,5). The presence of antihantavirus IgG was confirmed by immunofluorescent antibody (IFA) assay using whole hantavirus antigen and Western blot using hantavirus CL-1 strain (6,7). In further analysis, neutralization antibodies against SEOV strain SR-11 were detected by focus reduction neutralization test (8). The viral RNA, however, was not detectable in the acute-phase blood sample by reverse transcription-PCR (RT-PCR) (9). Other serologic tests were performed for dengue fever, typhoid fever, hepatitis B, and malaria; results of culture of blood and urine were negative for bacteria.

Following confirmation of the diagnosis, close contacts of the patient were investigated. Two family mem-

bers of the patient did not have any symptoms compatible with HFRS; their serum samples were tested and found negative for antihantavirus IgG. Because the patient was a nurse, possible nosocomial transmission and sources were also investigated and excluded. On the basis of the patient's strong history of exposure to rodents at home, further investigation focused on the domestic rodent population.

From October 14 through 16, 2008, 110 rodent traps were set within and surrounding the patient's house.

The total catch was 32 rodents, of which 16 were *Rattus norvegicus*, 7 *R. exulans*, 5 *R. argentiventer*, and 4 *Bandicota indica*, respectively. By using ELISA, IFA, and Western blot, antihantavirus IgG was detected in serum from 7 rats, of which 5 were *R. norvegicus*, 1 *R. argentiventer*, and 1 *B. indica*. Further analysis using RT-PCR identified 2 SEOV strains from *R. norvegicus* and *R. argentiventer* captured in the patient's house. The M segment of 1 identified SEOV strain (24D1208) was sequenced and compared with 22

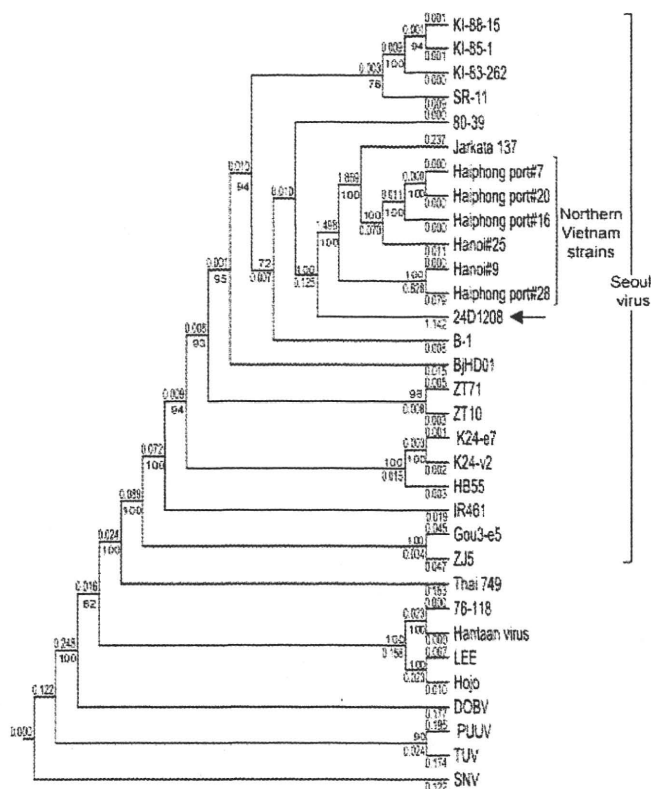


Figure. Phylogenetic tree (CLC-Combined Workbench 3) showing partial sequences of the medium segment (nt 810–2355). The newly identified Seoul virus (SEOV) was denoted as 24D1208 (arrow). The M segment sequences of the reference strains are: SEOV strains KI-88-15 (D17594), KI-85-1 (D17593), KI-83-262 (D17592), SR11 (M34882), 80-39 (S47716), Jakarta137 (AJ620583), Haiphong port #7 (AB355728), Haiphong port #20 (AB355730), Haiphong port #16 (AB355729), Hanoi #25 (AB355733), Hanoi #9 (AB355732), Haiphong port #28 (AB355731), B-1 (X53861), BjiHD01 (DQ133505), ZT71 (EF117248), ZT10 (DQ159911), K24-e7 (AF288652), K24-v2 (AF288654), HB55 (AF035832), IR461 (AF458104), Gou3-e5 (AF288650), and ZJ5 (FJ811839); Thailand virus strain 749 (L08756); Hantaan virus strains 76–118 (M14627), Hantaan (NC005219), LEE (D00377) and Hojo (D00376); Dobrava virus (DOBV) strain Dobrava (L33685); Puumala virus strain Sotkamo (X61034); Tula virus (TUV) strain Tula/Moravia/5302v/95 (Z69993); and Sin Nombre virus (SNV) strain NMH10. The numbers at the nodes are bootstrap confidence levels for 1,000 replications. Only bootstrap support values >70% are shown.

SEOV strains, 6 of which were from *R. norvegicus* rodents captured in urban areas of North Vietnam. Phylogenetic analysis showed that this SEOV belonged to the Vietnamese SEOV genotype (Figure).

We describe a clinical case of hantavirus infection and its potential rodent reservoir occurring in Vietnam. The clinical manifestations of the case-patient were compatible with SEOV infection, which is responsible for a moderate form of HFRS (10). Also, HFRS caused by SEOV occurs in urban rather than rural areas, unlike other hantavirus infections. Our epidemiologic findings were compatible with other studies indicating the source of infection was the case-patient's home, the only place where she had a history of exposure to rodents. Although viral RNA could not be obtained from the case-patient for genotyping, the genomic comparison of the viral strains from rodents captured in the case-patient's home and elsewhere in Vietnam suggested that the source of infection was local rodents. This report provides additional evidence that hantavirus infection is a worldwide problem and is likely underdiagnosed in Vietnam and other countries where simple standardized laboratory diagnostics are not widely available.

Acknowledgments

We thank Phan Ngoc Nam, Ha Van Loi, and Tran Luong Anh for their assistance in the case investigation. We also thank Rika Endo for her assistance in laboratory work and Nguyen Dac Tho and his team for their assistance in the rodent investigation.

This work was supported by a cooperative grant from the Pasteur Institute of Ho Chi Minh City, Vietnam, and Hokkaido University Graduate School of Medicine, Sapporo, Japan.

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DOI: 10.3201/eid1602.091204

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Origin of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus, China

To the Editor: A highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV), which affected >2 million pigs, emerged in early 2006 in the People's Republic of China. The disease was characterized by high fever (41°C), high illness rates (50%–100%), and high death rates (20%–100%) for pigs of all ages (1). A number of HP-PRRSVs have been isolated from 2006 through 2009 from infected pigs in different provinces of China and confirmed to be the causative agent of the new outbreaks (1,2). These HP-PRRSVs have a deletion of 30 amino acids in nonstructural protein 2 (NSP-2). However, the evolutionary origin and path of the HP-PRRSV remain unknown.

We analyzed the full-length sequences of 67 PRRSVs: 35 HP-PRRSVs (HuN4 and LNSY-08-1 isolated in our laboratory and 33 viruses isolated in other laboratories), 28 classic PRRSVs (18 viruses isolated from China and 10 viruses representing other Asian countries and North

Short Communication

Serological Evidence of Thailand Virus-Related Hantavirus Infection among Suspected Leptospirosis Patients in Kandy, Sri Lanka

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(Received August 30, 2010. Accepted December 3, 2010)

SUMMARY: A cross-sectional study was undertaken to determine the current prevalence of leptospirosis and hantaviral infections, and the socio-demographic characteristics and risk factors of infected patients, in Kandy, Sri Lanka. This report discusses the serological evidence of hantavirus infections among 105 suspected leptospirosis patients, 8 of whom had hantavirus antibodies. Serotyping ELISA showed that these 8 patients had high optical density values for Thailand virus. Most of the sera showed that the focus reduction neutralization test titer against Thailand virus was higher than that against Seoul virus, thereby suggesting that the hantaviral antibodies found in Sri Lanka are different from Seoul virus but closely related to Thailand virus. These findings imply that the hantaviral infection found in Kandy, Sri Lanka appears to be due to a virus similar to Thailand virus. Epidemiological analysis revealed that the association between hantavirus infection and socio-demographic characteristics was not statistically significant.

Hantavirus infections are an important zoonotic disease that cause either hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS) in humans. A significant number of people are affected with hantavirus worldwide (1), with approximately between 150,000–200,000 people throughout Asia and Europe being hospitalized with HFRS annually (2). Hantaviruses are negative-stranded RNA viruses in the genus *Hantavirus* of the family *Bunyaviridae* (1). Two species of hantaviruses, namely Seoul virus (SEOV) and Thailand virus (THAIV), have been reported as common causative agents of HFRS in south Asia (1).

Humans acquire the viruses primarily from inhaling aerosols contaminated with urine, feces, or saliva from chronically infected rodents (1). However, although hantavirus infections have been reported to occur among suspected leptospirosis patients (3), hantavirus infection and leptospirosis are indistinguishable in the absence of confirmatory diagnostic tests due to their similar clinical and epidemiological features.

Sri Lanka is one of the South Asian countries with the highest rates of leptospirosis notifications (4). Indeed, the number of cases notified increased four-fold (1,550 to 7,099 case notifications) between 2005 and 2008 (4).

However, most of these cases could not be confirmed due to the lack of laboratory facilities in the country. It is therefore not known whether these notifications were indeed leptospirosis or hantaviral infections. Although various studies have documented the existence of leptospirosis infections in both humans (5,6) and animals (7) in Sri Lanka, there have been limited local studies on hantavirus infection. A single laboratory investigation on local hantavirus infections conducted in 1988 found the presence of anti-hantavirus antibodies among suspected leptospirosis patients and the presence of hantavirus infections among *Rattus norvegicus*, a known reservoir for SEOV (8). This report discusses the most recent laboratory and epidemiological findings regarding hantavirus infections in Sri Lanka. Public health interventions need up-to-date and accurate information to prevent and control hantavirus infections effectively.

A cross-sectional study was undertaken between May and December 2008 to determine the current prevalence of leptospirosis and hantaviral infections, and to describe the socio-demographic characteristics and risk factors of patients having leptospirosis and/or hantaviral infection. The study included 107 patients from Kandy district who were admitted to the Peradeniya University Teaching Hospital in Kandy, Sri Lanka (Fig. 1). All patients had febrile disease of unknown origins and were identified as having leptospirosis on the basis of the surveillance case definition (4). Samples from suspected patients were examined using polymerase chain reaction (PCR) and the microscopic agglutination test, which comprises a battery of representative pathogenic leptospiral serogroups recommended by the World

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Health Organization. Leptospiral DNA was detected in 3 of the 107 serum samples, and anti-leptospiral antibodies were observed in 26 of 107 serum samples (24.3%) (6). This report discusses the serological evidence of hantavirus infections among suspected leptospirosis patients in Kandy, Sri Lanka. Information re-

garding the number of confirmed leptospirosis patients (29/107) has been published elsewhere.

Two of the 107 serum samples with anti-leptospiral antibodies were excluded due to lack of basic epidemiological information. The remaining 105 serum samples were screened for anti-hantaviral IgG antibodies using two tests. The first screening test was an indirect immunofluorescence antibody assay (IFA) using acetone-fixed smears of Vero E6 cells infected with Hantaan virus (HTNV), which shares some antigenic properties with hantavirus, as a primary antigen and fluorescent isothiocyanate (FITC)-conjugated goat anti-human IgG (Zymed, San Francisco, Calif., USA) as a secondary antibody. The second screening test was an enzyme-linked immunosorbent assay (ELISA) comprising the use of microtiter plates coated with a His-tagged recombinant nucleocapsid protein of HTNV and His-tag (3). Serotyping ELISA was carried out with the serum samples of IgG-positive participants using truncated nucleocapsid proteins of SEOV and THAIV together with whole HTNV nucleocapsid protein as antigens (9). A focus reduction neutralization test (FRNT) against SEOV and THAIV was also carried out for these sera, as described previously (3). The socio-demographic and environmental characteristics of the participants were collected and tabulated in Microsoft® Excel 2007. The refined Excel sheet was then exported to SPSS ver. 14 for subsequent statistical analysis.

Table 1 shows that about two-thirds of the 105 patients were aged 13–44 years, whereas the rest were older

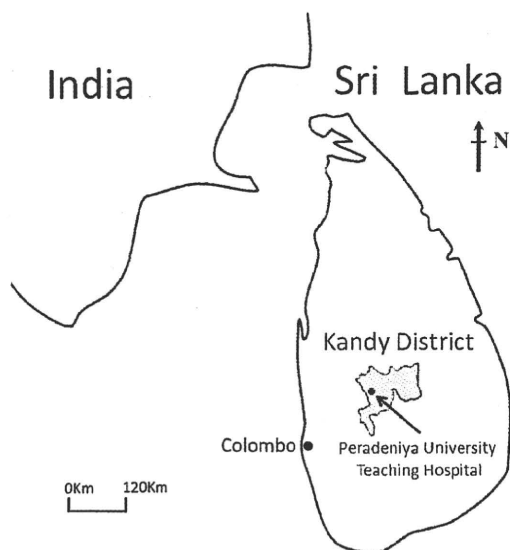


Fig. 1. Map of Sri Lanka, with Kandy district and Peradeniya University Teaching Hospital highlighted.

Table 1. General characteristics of serologically confirmed patients in the University of Peradeniya Teaching Hospital, Kandy, Sri Lanka

Characteristic	Total (n = 105)		No. positive patients (n = 8)		No. negative patients (n = 97)		Odds ratio (95% confidence interval)
	no.	%	no.	%	no.	%	
Age (y)							
13–44	70	66.7	3	4.3	67	95.7	0.27 (0.06–1.20)
45 or above	35	33.3	5	14.3	30	85.7	Reference
Gender							
Men	93	88.6	7	7.5	86	92.7	0.89 (0.10–7.98)
Women	12	11.4	1	8.3	11	91.7	Reference
Occupation (Farmer)							
Yes	25	23.8	3	12	22	88.0	2.04 (0.45–9.24)
No	80	76.2	5	6.3	75	93.7	Reference
Involvement in agriculture activities							
Yes	51	48.6	6	11.8	45	88.2	3.47 (0.67–18.04)
No	54	51.4	2	3.7	52	96.3	Reference
Interactions with domestic animals							
Dogs							
Yes	66	62.9	5	7.6	61	92.4	0.98 (0.22–4.36)
No	39	37.1	3	7.7	36	92.3	Reference
Cats							
Yes	67	63.8	7	10.4	60	89.6	4.32 (0.51–36.50)
No	38	36.2	1	2.6	37	97.4	Reference
Cattle							
Yes	76	72.4	7	9.2	69	90.8	2.84 (0.33–24.16)
No	29	27.6	1	3.4	28	96.6	Reference
Presence of peridomestic animals in one's surrounding or the residence							
Wild boar							
Yes	76	72.4	5	6.6	71	93.4	0.61 (0.14–2.74)
No	29	27.6	3	10.3	26	89.7	Reference
Rodents							
Yes	80	76.2	7	8.8	73	91.2	2.30 (0.27–19.67)
No	25	23.8	1	4.0	24	96.0	Reference

Table 2. Distribution of leptospirosis and anti-hantaviral antibodies among hospitalized patients in the Peradeniya Teaching Hospital, Kandy, Sri Lanka ($n = 105$)

		Anti-hantaviral antibodies ²⁾		Total
		Positive	Negative	
Leptospirosis ¹⁾	Positive	3	24	27
	Negative	5	73	78
	Total	8	97	105

¹⁾: Results from the published report by Koizumi et al. (6). Among 29 positive patients of leptospirosis, three were detected by *flaB*-PCR assay and 26 by micro agglutination test (MAT). Two MAT positive cases were excluded in this study due to lack of epidemiological data.

²⁾: Serologically tested by IFA (1:100) and ELISA (1:200). In this table, IFA positive and ELISA positive patients were recognized as anti-hantaviral antibody positive result. Hantaan virus strain 76118 infected Vero E6 cell smears were used as antigen for IFA assay. Recombinant protein of 103 amino acid of N-terminal region of N protein of Hantaan virus strain 76118 was expressed as fusion protein with Nus protein in *Escherichia coli* by using pET43.1 vector (Novagen) as ELISA antigen.

(mean age, 41 years; SD \pm 14.42). The overwhelming majority of patients (88.6%) were male, and three-fourths (76.2%) were non-farmers. The patients were essentially equally divided into those with and without involvement in agricultural activities (48.6% versus 51.4%). Roughly two-thirds (62.9–63.8%) and three-fourths (72.4–76.2%) of patients had some form of contact with domestic and peridomestic animals, respectively. A descriptive analysis of the socio-demographic characteristics and risk factors of those patients who tested positive for hantavirus antibodies revealed that patients tended to be over 45 years of age, were involved in agricultural activities, and had come into contact with domestic and/or peridomestic animals. However, further analysis revealed that this association between hantavirus infection and socio-demographic characteristics was not statistically significant.

Table 2 highlights the number of patients with anti-hantaviral antibodies. Eight of the 105 patients had the anti-hantaviral antibodies, and three were also positive for leptospirosis; 73 were negative for both leptospirosis and hantaviral infection. The statistical analysis revealed no association between having leptospirosis and having positive hantavirus antibodies (or past history of hantavirus infections) (OR = 1.825; 95% CI = 0.448–7.518). The results from the serotyping ELISA showed that the eight patients had high OD values for THAIV (Fig. 2). Further, most of the sera had a higher FRNT titer against THAIV than against SEOV, although the difference was less than four-fold except for one positive serum. The hantavirus infection found in Sri Lanka therefore seems to differ from SEOV but to be closely related to THAIV. These findings suggest that the hantavirus antibodies detected in this study in Kandy, Sri Lanka appear to have been induced by a THAIV-related hantavirus infection.

THAIV was originally isolated from a greater bandicoot rat (*Bandicota indica*) captured in Thailand (10). Hantaviruses are believed to coevolve with their host rodents (11). Two bandicoot rat species, namely *B. indica* and *B. bengalensis*, which might serve as potential candidates for the host rodents of THAIV-related virus, are

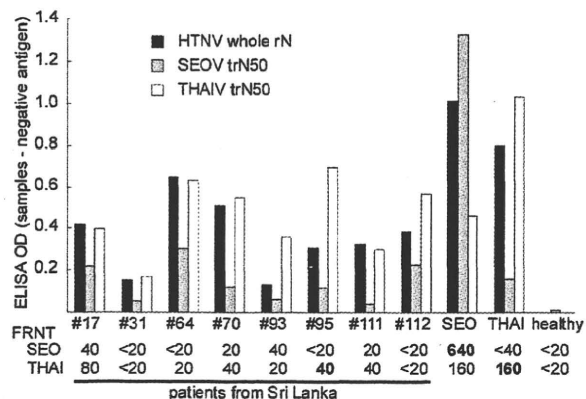


Fig. 2. Serotyping analysis of anti-hantavirus positive sera from Kandy, Sri Lanka. Eight sera were anti-hantavirus positive in screening tests (IFA [1:100] and ELISA [1:200]). The positive controls of Seoul (SEO) and Thailand (THAI) virus antibodies were obtained from laboratory outbreak, Japanese and Thai patient (4), respectively. Negative control was obtained from clinically healthy individual. Black bars indicate ELISA ODs against whole N antigen of Hantaan virus (HNTV) with common antigenic region. Grey bars and white bars indicate ELISA ODs against serotyping antigen of SEOV and THAIV, respectively. The focus reduction neutralization test (FRNT) was expressed as the reciprocal of the highest serum dilution that resulted in a >80% reduction in the number of infected cell foci. FRNT titers against SEOV and THAIV indicated under serum ID.

known in Sri Lanka (12). Furthermore, as both leptospirosis and hantavirus infections are rodent-borne zoonoses, the infection risks for these two agents could overlap, possibly as a result of the exposure of patients to wild animals and/or their excreta.

Anti-THAIV antibodies have previously been detected in suspected leptospirosis patients in Thailand (3) and India (13). Herein we also demonstrated the presence of THAIV-related virus infection in humans in Sri Lanka. Most patients in the study had low IgG antibody titers and lacked IgM antibody (data not shown), thus suggesting a prior infection. The acute forms of leptospirosis and hantaviral infections possess very similar clinical symptoms (2), thus meaning that they are difficult to differentiate without isolating the pathogen, demonstrating the presence of IgM antibodies, or an increased IgG titer. It should be noted that none of the 105 patients had an acute hantavirus infection. It is therefore important to upgrade laboratory facilities to distinguish hantaviral antibody positive patients from acute hantaviral infections. Since this study offers limited epidemiological data, it is difficult to explain the disease dynamics and pathogenicity of THAIV in humans. In addition, despite their febrile status, 73 out of the 105 patients were diagnosed as having neither anti-leptospira antibodies nor anti-hantaviral antibodies. Further laboratory diagnoses are therefore required to determine the etiology of the febrile disease.

In summary, we have detected the occurrence of THAIV-related hantavirus infections among suspected leptospirosis patients in Kandy, Sri Lanka, thus suggesting that hantavirus infection may be an underreported and emerging disease in this country. Systematic laboratory diagnosis and surveillance should therefore be undertaken to gain an understanding of the epidemiology