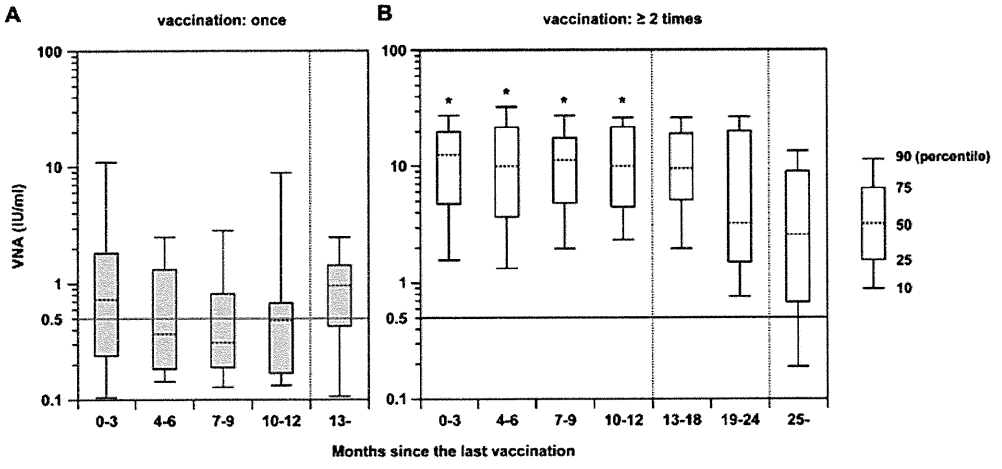

C

Vaccination	once					≥ 2 times						
Months since the last vaccination	0-3	4-6	7-9	10-12	13-	0-3	4-6	7-9	10-12	13-18	19-24	25-
Total no.	26	24	15	14	13	74	83	51	172	145	13	19
No. VNA ≥ 0.5 IU/ml (%)	15 (57.7)	10 (41.7)	5 (33.3)	7 (50.0)	10 (76.9)	72 (97.3)	81 (97.6)	50 (98.0)	172 (100)	142 (97.9)	13 (100)	15 (78.9)
GM of VNA level (IU/ml)	0.83	0.50	0.43	0.61	0.69	8.32	7.56	8.17	8.78	8.37	5.05	2.12

Fig. 1. Duration of protective VNA levels in dogs after rabies vaccinations. Box and whisker plots of VNA during each period after the last vaccination of dogs that had been singly vaccinated (A) or multiply vaccinated (B). Asterisks indicate significant differences in the VNA levels of dogs that had received a single vaccination in the corresponding period since the last vaccination ($P < 0.01$, Mann-Whitney U test). (C) This table lists the characteristics of the protective VNA duration in dogs. The number of dogs with VNA measuring ≥0.5 IU/ml and the corresponding geometric mean (GM) of VNA during each period are indicated.

Table 2. Details of 12 dogs that received multiple vaccinations but had inadequate VNA levels

	Dog breed	Sex	Date of birth	Age (yr)	No. of vaccinations	Days after vaccination	VNA (IU/ml)
1	Miniature Dachshund	Male	5/1/2005	5	3	76	0.40
2	Labrador Retriever	Female	4/22/2001	10	11	77	0.35
3	Japanese Kishu	Female	1/1/2003	8	5	98	0.26
4	Mixed	Female	4/1/2008	2	3	122	0.35
5	Mixed	Female	6/1/1996	14	15	214	0.40
6	Scottish Terrier	Female	4/1/2003	7	4	377	0.19
7	Miniature Dachshund	Male	5/7/2001	10	7	404	0.48
8	Mixed	Female	4/10/2009	1	2	445	0.46
9	Miniature Dachshund	Not known	9/1/1997	13	5	745	0.14
10	Cairn Terrier	Male	7/15/1999	11	7	1,252	0.27
11	Mixed	Male	1/11/2001	10	2	1,281	0.31
12	Labrador Retriever	Male	8/14/2005	5	2	1,329	0.13

(i.e., 7.56–8.78 IU/ml). Within each time period after the last vaccination (0–3, 4–6, 7–9, or 10–12 months), VNA levels differed significantly in dogs that received single versus multiple vaccinations. In contrast, VNA levels in multiply vaccinated dogs declined by 25 months following the last vaccination. The geometric mean of VNA in this group was 2.12 IU/ml, and 78.9% of those dogs maintained protective VNA levels. These data indicate that the VNA levels were maintained adequately for 2 years following the last vaccination in dogs that had been vaccinated at least twice.

Of the 557 multiply vaccinated dogs, 12 lacked protective VNA levels. The characteristics of the dogs with inadequate VNA levels are listed in Table 2 ordered according to most recent vaccination. Of this group, 5 serum samples (Nos. 1–5) were collected less than 1 year following the last vaccination, whereas 7 samples (Nos. 6–12) were collected more than 1 year after the last vaccination. None of the dogs in this study had an immune disorder. No direct correlations were observed between lower VNA levels and sex (Spearman's rank correlation coefficient test).

Potential maternal antibody transferred in unvaccinated dogs: Of the 107 serum samples collected from unvaccinated dogs, 72 were obtained from dogs aged 90 days or less and 35 were obtained from dogs aged

Table 3. VNA levels in unvaccinated dogs

Age range (days)	No. of samples	VNA (IU/ml)	
		<0.25	≥0.25
1-90	72	61	11
91-365	35	34	1

91-365 days. As described above, we found that only 1 unvaccinated dog exhibited protective VNA levels whereas many unvaccinated dogs exhibited inadequate VNA levels. We considered the possibility that the VNA detected in unvaccinated puppies derived from maternally antibody transfer; however, the levels of antibody were insufficient for protectection against rabies. To examine potential maternal transfer of antibody, we set the VNA level of 0.25 IU/ml as the cutoff value. The VNA levels in these puppies are listed in Table 3. Of the 72 unvaccinated puppies aged 90 days or less, 11 samples (15.3%) exhibited VNA levels of ≥0.25 IU/ml. In contrast, only 1 of the 35 unvaccinated puppies aged over 90 days (2.9%) exhibited VNA levels of ≥0.25 IU/ml.

DISCUSSION

The WHO recommends that at least 70% of the dog population should be immunized against rabies to minimize the risk of its reemergence (1), and this recommendation is particularly important in Japan, where rabies has been eradicated. A mass rabies vaccination of dogs is conducted in Japan each year, but neither the current status of rabies VNA in dogs nor the rationale for conducting an annual rabies vaccination program has been assessed. Therefore, we aimed to determine the actual VNA levels in dogs that had visited animal hospitals in Japan.

We determined that approximately 50% of singly vaccinated dogs did not exhibit protective levels of serum VNA within 1 year of vaccination, which indicates that single vaccination dosing is insufficient for establishing herd immunity. A previous study also demonstrated that the VNA titer dropped to <0.5 IU/ml in >50% of dogs vaccinated within 120 days after a single vaccination (11). In the current study, however, the VNA levels in dogs that had received a single vaccination were higher 13 months after a single vaccination than that within 12 months of vaccination (Figs. 1A and 1C). The reason for this finding is unclear; however, our study differed from others (11) because we obtained serum samples from different individuals (i.e., not from the same dogs at different time points). In addition, we observed no significant differences in VNA levels ($P > 0.05$, Mann-Whitney U test) in dogs 13 months after vaccination and those of dogs during each period within 12 months of vaccination.

In contrast, most dogs that were vaccinated at least twice demonstrated protective VNA levels; the VNA in these dogs was significantly higher than that in singly vaccinated dogs. Our results are consistent with previous reports (6,12). We also found that protective VNA levels persisted 2 years after the last vaccination in most multiply vaccinated dogs. Of the 557 dogs that received at least 2 vaccinations, only 12 (2.2%) did not exhibit protective VNA levels; it was assumed that these dogs were low responders to the vaccine and/or their levels had declined over time. However, this did not pose an issue because the number was very low, and mass dog vaccination aims to achieve herd immunity for protection of human public health. Overall, the current mass rabies vaccination program in Japan should provide adequate herd immunity if most Japanese dogs are registered and vaccinated.

We confirmed that protective VNA levels persisted for 2 years after the last vaccination in dogs with a history of at least 2 vaccinations (Fig. 1B), which indicates that the current annual vaccination schedule is sufficient for protection against rabies, although, a biennial vaccination schedule could be considered. In Texas, the vaccination schedule has shifted from yearly to triennial and effectively increased dog and cat vaccination rates significantly (13). Several factors require careful consideration if the mandatory vaccination schedule in Japan is changed in the future, including confirmation of sustainable VNA levels, the introduction of a simple serological survey method, and measures implemented to protect low responders. We recommend that VNA levels in dogs are checked every year to confirm the need for vaccination. However, the methods used to measure the rabies VNA level, such as virus neutralization assays and ELISA, are inconvenient and require specialized laboratories and equipment, and trained personnel. Recently, we developed a rapid, safe, easy, and inexpensive VNA test for rabies based on a combination of an immunochromatographic technique and a competitive immunoassay with neutralizing monoclonal antibodies (14,15). This semiquantitative test determines within 60 min whether a dog has protective VNA levels, without using a live virus and expensive equipment. This may represent a convenient method for determining whether dogs require booster immunization.

The Ministry of Health, Labour and Welfare of Japan reported the registration of 6.88 million dogs in 2009; 74.3% had been vaccinated against rabies (16). However, in 2009, the Japan Pet Food Association reported that the number of breeding dogs in Japan was 12.32 million (17). When unregistered dogs are considered in the total, only 41.5% of dogs are vaccinated against rabies, which pushes the immunization level far below the required 70% level recommended for rabies epidemics prevention in a population. The Ministry of Agriculture, Forestry, and Fisheries of Japan reported that about 5 million doses of rabies vaccine were produced in 2011 (18), which indicates that insufficient vaccine availability to cover all dogs in Japan. Therefore, we suggest in the event of an outbreak of rabies in Japan, unvaccinated dogs or singly vaccinated dogs should receive vaccine priority above dogs that have been vaccinated at least twice.

In Japan, about 50,000 stray dogs visit animal health centers each year (16) and approximately 5,000 dog bite cases are reported (19). Unvaccinated stray dogs may be a risk for the circulation of rabies and its transmission to humans. An understanding of rabies seroprevalence in stray dogs is important for an accurate assessment of the risk of rabies reemergence in Japan, but its seroprevalence was not surveyed in this study. Ogawa et

al. reported that only 27.7% of stray dogs that were captured by rabies prevention officers and brought in to the Hyogo Prefecture Animal Well-being Center in Japan in 2006 and 2007 exhibited protective VNA levels (20). Additional seroprevalence surveys should be conducted on stray dogs from other geographical areas to determine the actual state of rabies immunity in the dog population of Japan.

We detected maternal rabies antibodies in 15.3% of unvaccinated puppies less than 3 months in age, whereas we detected these antibodies in only 1 unvaccinated dog 3 or more months in age. The rabies vaccines that are produced for dogs in Japan are inactivated, and thus, maternal antibodies do not interfere with the vaccine response in puppies (21). However, maternal antibodies can interfere with the immune response to live attenuated rabies vaccines (22,23). Therefore, the future introduction of live vaccines for mass dog immunization should consider the timing of vaccinations in puppies.

The results of this study suggest the current annual rabies dog vaccination program is adequate given that all dogs are registered and vaccinated each year. However, as suggested above, the rates of rabies vaccination in dogs of Japan are supposed to be far less than 70%. More than 60 years have passed since the introduction of the Rabies Prevention Law in Japan, and Japanese lifestyles and the relationships between humans and pet dogs have changed dramatically during this period. Therefore, the present may be the appropriate time period to consider updating the rabies prevention system.

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Conflict of interest None to declare.

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A survey of rodent-borne pathogens carried by wild *Rattus* spp. in Northern Vietnam

T. KOMA[1], K. YOSHIMATSU[1], S. P. YASUDA[1], T. LI[2], T. AMADA[1], K. SHIMIZU[1], R. ISOZUMI[1], L. T. Q. MAI[3], N. T. HOA[3], V. NGUYEN[4], T. YAMASHIRO[5,6], F. HASEBE[7] AND J. ARIKAWA[1]*

[1] *Department of Microbiology, Graduate School of Medicine, Hokkaido University, Japan*
[2] *Department of Virology 2, National Institute of Infectious Diseases, Japan*
[3] *National Institute of Hygiene and Epidemiology, Hanoi, Vietnam*
[4] *Centre for International Health Quarantine, Hai Phong City, Hai Phong, Vietnam*
[5] *Institute of Tropical Medicine, Nagasaki University, Japan*
[6] *Vietnam Research Station, Nagasaki University, Japan Initiative for Global Research Network on Infectious Diseases (J-GRID)*
[7] *Centre of International Collaborative Research, Nagasaki University, Japan*

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SUMMARY

To examine the prevalence of human pathogens carried by rats in urban areas in Hanoi and Hai Phong, Vietnam, we live-trapped 100 rats in January 2011 and screened them for a panel of bacteria and viruses. Antibodies against *Leptospira interrogans* (22·0%), Seoul virus (14·0%) and rat hepatitis E virus (23·0%) were detected in rats, but antibodies against *Yersinia pestis* were not detected. Antibodies against *L. interrogans* and Seoul virus were found only in adult rats. In contrast, antibodies to rat hepatitis E virus were also found in juvenile and sub-adult rats, indicating that the transmission mode of rat hepatitis E virus is different from that of *L. interrogans* and Seoul virus. Moreover, phylogenetic analyses of the S and M segments of Seoul viruses found in *Rattus norvegicus* showed that Seoul viruses from Hai Phong and Hanoi formed different clades. Human exposure to these pathogens has become a significant public health concern.

Key words: Hantavirus, hepatitis E, leptospirosis, surveillance, zoonoses.

INTRODUCTION

Rodents play a role as reservoir hosts of causative agents for various bacterial, viral and parasitic zoonoses. Wild rats (*Rattus* spp.) are a particularly important source of human pathogens because they inhabit areas in the vicinity of human dwellings.

* Author for correspondence: Professor J. Arikawa, Department of Microbiology, Graduate School of Medicine, Hokkaido University, Kita-ku, Kita-15, Nishi-7, Sapporo 060–8638, Japan. (Email: j_arika@med.hokudai.ac.jp)

Leptospirosis is caused by spirochaetes belonging to the genus *Leptospira*. Leptospirosis is an important worldwide zoonosis for which the major reservoir animals are rodents. Although some leptospirosis cases have been diagnosed correctly, leptospirosis is thought to be a major cause of undiagnosed acute febrile illness (AFI) in endemic countries [1]. About 12 outer membrane proteins, including LipL32, OmpL1, Ligb, LenA, LenD and Loa22, have been identified [2]. The major outer membrane lipoprotein, LipL32, is the most abundant protein of the entire cell and is highly conserved in pathogenic *Leptospira* spp. [3].

Hantavirus infection, haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) are also known as worldwide rodent-borne viral zoonoses. Hantaviruses are enveloped and negative-sense RNA viruses with a tripartite genome comprising of large (L), medium (M) and small (S) segments [4]. Seoul virus (SEOV) is one of the causative agents of HFRS and is carried by *Rattus norvegicus*. We previously conducted epidemiological studies on the prevalence of SEOV infection in rodents and AFI patients who were not leptospirosis patients in East Asian countries including Vietnam [5, 6], Indonesia (Ibrahim *et al.*, unpublished data), Thailand [7], and Sri Lanka [8]. The epidemiological results indicate that SEOV infection exists in rodents and non-leptospirosis patients in all of those countries. However, hantavirus antibody-positive rates in non-leptospirosis AFI patients were about 2·3%, which is almost the same as the rate in healthy people in Vietnam [5]. Therefore, although SEOV infection is one of the possible causes of AFI, other causative agents are thought to exist.

Hepatitis E virus (HEV) is a positive-sense, single-stranded RNA virus, and the HEV genome includes two short non-coding regions surrounding three open reading frames (ORF1–ORF3). HEV can generally be divided phylogenetically into four genotypes. A genetically distinct HEV has recently been isolated from rats [9]. Our previous study showed the prevalence of rat HEV antibody in wild rats in Hanoi, Vietnam. Furthermore, the rat HEV genome was seen to be closely associated with rat HEV in Germany [10]. Although a recent study provided evidence of human infection with rat HEV in Germany [11], the relationship between rat HEV and human disease is still unclear.

In this study, we investigated the prevalence of *Leptospira interrogans*, SEOV and rat HEV in urban rats captured in urban areas in Hai Phong and Hanoi City, which are the second and third most populous cities in Vietnam, respectively. Antibody against *Yersinia pestis* was also examined as it is an important rodent-borne pathogen.

METHODS

Animals

Two hundred and 220 tomahawk live-traps were set in the evening and checked in the morning after a trapping night in residential districts of urban areas in Hanoi City (+105·84° E, 20·97° N) and inside a warehouse facing the residential district of an urban area in Hai Phong Port (+106·69° E, 20·87° N), in Northern Vietnam in January 2011. A total of 100 small mammals (94 *R. norvegicus*, 6 *R. tanezumi*) were captured in Hanoi City and in Hai Phong Port. Weight, sex and species identification were recorded for each animal. Species were identified by external morphology and DNA sequencing of the mitochondrial cytochrome *b* gene as described previously [5, 12]. Sequence data for cytochrome *b* obtained in this study were deposited in DDBJ/EMBL/GenBank (accession numbers: AB674753–AB674758, AB746356–AB746367). To investigate the relationship between maturation stage and seroprevalence, *R. norvegicus* were tentatively categorized by weight as juveniles (<100 g), sub-adults (100–200 g) and adults (>200 g) [13]. A blood sample was collected from each rodent via cardiac puncture under ether anaesthesia. Serum specimens were stored at −80 °C until serological examination. Lung specimens were collected and stored at −80 °C for polymerase chain reaction (PCR) examination of the hantavirus genome.

Antibody detection

Antibody against *L. interrogans* was detected by an enzyme-linked immunosorbent assay (ELISA) with *Escherichia coli*-expressed LipL32 of *L. interrogans* as an antigen according to a previously described method [14]. ELISA was performed essentially by the same procedure as described previously for hantavirus infection [15]. Briefly, wells of a 96-well plate were coated with 1 μg/ml antigen in phosphate-buffered saline (PBS). The plates were then blocked with PBS containing 3% bovine serum albumin (BSA) for 1 h at 37 °C. Rodent sera were diluted 1:200 with ELISA buffer (PBS containing 0·5% BSA and 0·05% Tween-20) and added to the wells. After incubation for 1 h at room temperature, the plates were washed three times with ELISA buffer, and horseradish peroxidase-conjugated goat anti-rat IgG antibody (Zymed Laboratories Inc., USA) was added as the secondary antibody. After incubation for 1 h at room temperature, the plates were washed as described above and colorimetric reaction was developed by the addition of *o*-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich, USA). Optical density (OD) was measured at 450 nm. A negative antigen that included the Nus-tag protein, made from the pET43b

vector, was used as a negative control. The sample OD was calculated by subtracting the average OD for each set of negative antigen duplicates from the average OD for each set of LipL32 duplicates. Serum samples from three wild rats (*R. norvegicus*), which had been confirmed as antibody-negative to *L. interrogans* by various diagnoses, were used as negative control sera. The negative control sera were examined in all ELISA experiments, and mean OD value plus three times the standard deviations (S.D.) was used as a cut-off value to distinguish ELISA-positive and ELISA-negative samples. Antibody-positive sera were then subjected to Western blotting (WB) using the same antigen by the procedure described previously [16]. Samples were considered *L. interrogans* IgG antibody-positive if they were positive by both ELISA and WB.

Antibody to SEOV was detected by IgG ELISA with *E. coli*-expressed N-terminal 103 amino acids of the nucleocapsid protein as an antigen (HS103) as described previously [7]. Wells of a 96-well plate were coated with 1 μg/ml HS103 in PBS as a capture antigen. ELISA using HS103 antigen was performed using the same procedure as described above. Antibody-positive sera were then subjected to WB using the baculovirus-expressed antigen using the same procedure as described above [17]. Samples were considered SEOV IgG antibody positive if they were positive by both ELISA and WB.

Antibody to rat HEV was detected by ELISA with virus-like particles consisting of baculovirus-expressed rat HEV ORF2 proteins as described previously [10]. Wells of a 96-well plate were coated with 1 μg/ml rat HEV ORF2 proteins in PBS as a capture antigen. ELISA was then performed essentially by the same procedure as described above. However, blocking was performed using 5% skimmed milk dissolved in PBS-T for 1 h at 37 °C.

Antibody to *Y. pestis* was detected by ELISA with Fraction 1 antigen, which is a capsule-like antigen encoded by the *caf1* gene, as described previously [18]. Wells of a 96-well plate were coated with 1 μg/ml Fraction 1 antigen in PBS as a capture antigen. ELISA was then performed using the same procedure as described above.

Molecular characterization of hantaviruses

Total RNA was extracted from lung tissues of all *R. norvegicus* and *R. tanezumi* rodents using Isogen (Nippon Gene, Japan) and then reverse-transcribed using a First-Strand cDNA Synthesis kit (GE Healthcare UK Ltd, UK). Real-time PCR followed by PCR for sequencing of the hantavirus genome were performed to amplify the target sequence. Primer and probe sequences for real-time PCR were as follows: Realtime SEOS F (5′-TATGGTTGC-CTGGGGAAAG-3′), Realtime SEOS R (5′-GCT-CTGGATCCATGTCATCA-3′) and probe no. 86 (5′-GCAGTGGA-3′). Hantavirus sequences were then amplified by PCR using primers for the S and M segments as follows: MurS110F (5′-CAGAAGG-TIAIGGATGCAGA-3′), SEOS1589R (5′-ACTTA-AGGTGACCTGGCCCT-3′), SEOM1277F (5′-TT-TAGAGCAGCTGAGCAGCAGAT-3′) and M12–3161R (5′-AACCACTATGGCCACCTTTC-3′).

PCR products were purified and DNA sequencing was performed as described previously [5]. Phylogenetic relationships among the hantavirus sequences were evaluated using the Neighbour-Joining (NJ) program with the Kimura two-parameter distance in CLUSTALW version 1.83 (European Bioinformatics Institute, UK). The phylogenetic tree was visualized using the NJ plot program. Bootstrap resampling analysis was performed using 1000 replicates.

The viral sequence data obtained in this study were deposited in DDBJ/EMBL/GenBank (accession numbers: AB674759–AB674769).

Statistical analysis

Differences between seroprevalence and body weight were examined for statistical significance by the Mann–Whitney U test. P values $<0{\cdot}05$ and $<0{\cdot}01$ were considered significant. Differences in seroprevalence, sex and geographical origin were examined for statistical significance by Pearson's χ^2 test or Fisher's exact test. To estimate the relationship of co-infection of a human pathogen in the rodents, we obtained an estimate from each study of the odds ratio (OR) with 95% confidence interval (95% CI).

RESULTS

Prevalence of antibodies to rodent-borne pathogens

The trapping rates of rodents in Hanoi City and Hai Phong Port were 32·0% and 15·0%, respectively. Prevalence of antibodies against four rodent-borne pathogens is given in Table 1. Antibodies against *L. interogans* were detected in 21·7% (13/60) and 26·5% (9/34) of *R. norvegicus* captured at Hanoi City

Table 1. *Trapping sites, collected rodent species, and seropositivity for* L. interrogans, *SEOV, rat HEV and* Y. pestis

Location	Species	No. tested	No. seropositive against (% positive)			
			*L. interrogans**	SEOV*	Rat HEV†	*Y. pestis*†
Hanoi City	*R. norvegicus*	60	13 (21·7%)	3 (5%)	10 (16·7%)	0 (0%)
	R. tanezumi	4	0 (0%)	0 (0%)	2 (50%)	0 (0%)
Hai Phong Port	*R. norvegicus*	34	9 (26·5%)	11 (32·4%)	11 (32·4%)	0 (0%)
	R. tanezumi	2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total		100	22	14	23	0

SEOV, Seoul virus; HEV, hepatitis E virus.
* Seroprevalence determined by ELISA and Western blotting.
† Seroprevalence determined by ELISA.

and Hai Phong Port, respectively ($P=0·60$). SEOV antibody-positive *R. norvegicus* were obtained both in Hanoi City and Hai Phong Port, but the positive rate was higher in Hai Phong (32·4%, 11/34) than in Hanoi (5%, 3/60) ($P<0·01$). There were no *R. tanezumi* seropositive to *L. interogans* and SEOV. Prevalence of antibodies against rat HEV were 16·7% (10/60) in *R. norvegius* and 50% (2/4) in *R. tanezumi* captured in Hanoi and 32·4% (11/34) *R. norvegicus* captured in Hai Phong. There was no significant difference between rat HEV prevalence rates of *R. norvegicus* in Hanoi and Hai Phong ($P=0·08$). There were no rats seropositive to *Y. pestis*.

Body weight, geographical origin, sex and co-infection

The mean body weight of *R. norvegicus* in Hai Phong was significantly greater than that of *R. norvegicus* in Hanoi (336·4 g *vs.* 289·4 g, $P<0·05$). However, no significant difference in sex or rate of maturation stage (juvenile and sub-adults *vs.* adults) of *R. norvegicus* was found between rats captured in Hai Phong and rats captured in Hanoi ($P=0·72$ and $P=0·78$, respectively). The mean body weight of *R. norvegicus* infected with SEOV was significantly greater in both males and females than in SEOV-negative rats (Fig. 1). *L. interrogans* antibody-positive females were also significantly heavier than uninfected female rats. However, no significant body-weight difference was found between *L. interrogans* antibody-positive males and antibody-negative males. No significant body-weight difference was found between HEV-infected rats and uninfected rats.

All of the rats with antibodies against *L. interrogans* and SEOV were adult *R. norvegicus* with body weights of >260 g and >340 g, respectively. On the other hand, rat HEV antibody-positive *R. norvegicus* were found in juveniles and sub-adults: one male (84 g) and 4/15 females (100–170 g). There was no infant rat with maternal antibody.

Although no significant difference was found, female *R. norvegicus* tended to be more frequently infected than males with *L. interrogans* (28·3% *vs.* 17·1%, $P=0·20$), SEOV (17·0% *vs.* 12·2%, $P=0·52$) and rat HEV (28·3% *vs.* 14·6%, $P=0·11$).

ORs were calculated in seropositive rats to examine the particular combination of co-infection. ORs of co-infection with *L. interrogans* and SEOV, SEOV and rat HEV, and *L. interrogans* and rat HEV were 2·0 (95% CI 0·56–6·70, $P=0·20$), 1·4 (95% CI 0·35–4·89, $P=0·64$), and 1·4 (95% CI 0·43–4·04, $P=0·64$), respectively. Thus, no significant ORs were obtained in any combination.

Molecular characterization of SEOV

Lung specimens of all *R. norvegicus* and *R. tanezumi* were examined for their virus genome by real-time PCR. All but two of the specimens from seropositive rats were positive by real-time PCR. No real-time PCR-positive specimen was obtained from sero-negative rats.

Based on the real-time PCR results, six of the specimens that showed strong positivity were selected and subjected to reverse transcriptase-PCR. Finally, five S-segment sequences and six M-segment sequences were successfully recovered. The phylogenetic trees were drawn using 1378 nt (194–1571) of the S segment and 1101 nt (1966–3066) of the M segment (Fig. 2). All of the sequences were included in the SEOV clade both in the S-segment and M-segment phylogenetic trees. SEOV from Hai Phong

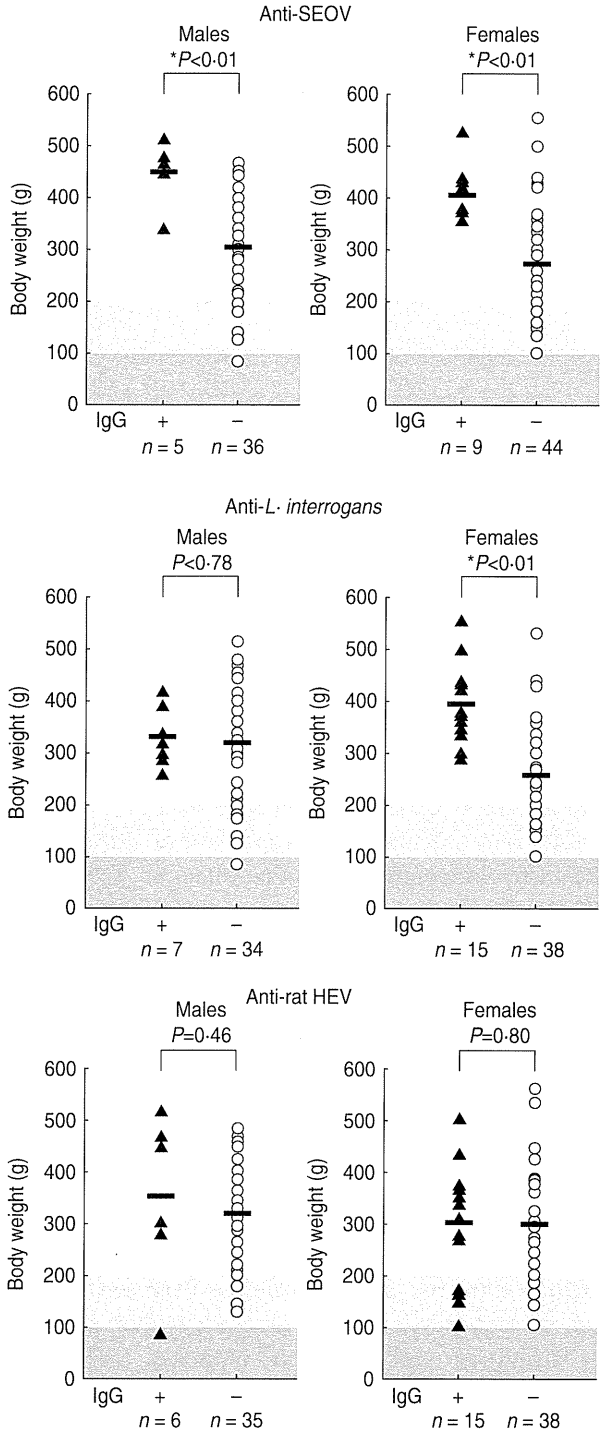

Fig. 1. Relationship between seroprevalence and body weight in *R. norvegicus*. Solid line indicates mean body weight. The grey and light-grey shaded areas indicate juveniles (< 100 g) and sub-adults (100–200 g), respectively. An asterisk indicates statistical significance at $P < 0{\cdot}01$.

(HaiPhong3-11, 17-11, 24-11, 31-11), and SEOV from Southern Vietnam (5CSG and 11CSG), formed one group, which was separated from the other group consisting of SEOV from Hanoi and Singapore.

DISCUSSION

The present study provides information regarding the prevalence of *L. interrogans*, SEOV and HEV in wild rats in urban areas in Hanoi City and Hai Phong Port in Northern Vietnam. The seroprevalence of *L. interrogans* and rat HEV in rats was high in both cities. Various prevalence rates of *Leptospira* and hantavirus infection in wild *Rattus* spp. have been reported in several countries in Asia: reported prevalence rates of *Leptospira* were 5–30% [19–25] and those of hantavirus were 5–20% [5, 26–30]. Our results regarding the seroprevalence of *L. interrogans* and SEOV are consistent with those of previously reported studies. Therefore, our results confirm the potential hazard to humans. A recent study provided evidence for the presence of anti-rat HEV IgG in forestry workers in Germany [11]. However, the relationship between rat HEV and human disease is still unclear. Therefore, further seroepidemiological studies in cryptogenic hepatitis patients should be conducted.

Although the relationship between each seroprevalence and body weight in *R. norvegicus* was reanalysed with the entry of geographical information to discover the relationship to geographical origin, the mean body weight of male *R. norvegicus* infected with SEOV, *L. interrogans* and rat HEV both in Hanoi and Hai Phong was not significantly different from the mean body weight of uninfected rats ($P > 0{\cdot}09$) (data not shown). On the other hand, the mean body weight of female *R. norvegicus* infected with SEOV and *L. interrogans* both in Hanoi and Hai Phong was significantly greater than the mean body weight of uninfected rats ($P < 0{\cdot}05$) (data not shown). Female *R. norvegicus* infected with rat HEV in Hanoi tended to be heavier than uninfected rats (340·4 g *vs.* 261·7 g, $P = 0{\cdot}08$). Interestingly, rat HEV antibody-positive female *R. norvegicus* in Hai Phong were lighter than uninfected female rats (261·8 g *vs.* 363·6 g, $P < 0{\cdot}05$). However, the reason for the inverse correlation between body weight of rat HEV antibody-positive female *R. norvegicus* in Hai Phong and Hanoi is unclear. Further longitudinal studies are needed to clarify the relationships regarding geographical origin, sex and weight factors.

L. interrogans and SEOV were detected only in adult *R. norvegicus* with body weights of > 260 g and > 340 g, respectively. Furthermore, antibody-positive rates increased with weight (age), suggesting that *L. interrogans* and SEOV are maintained in reservoir

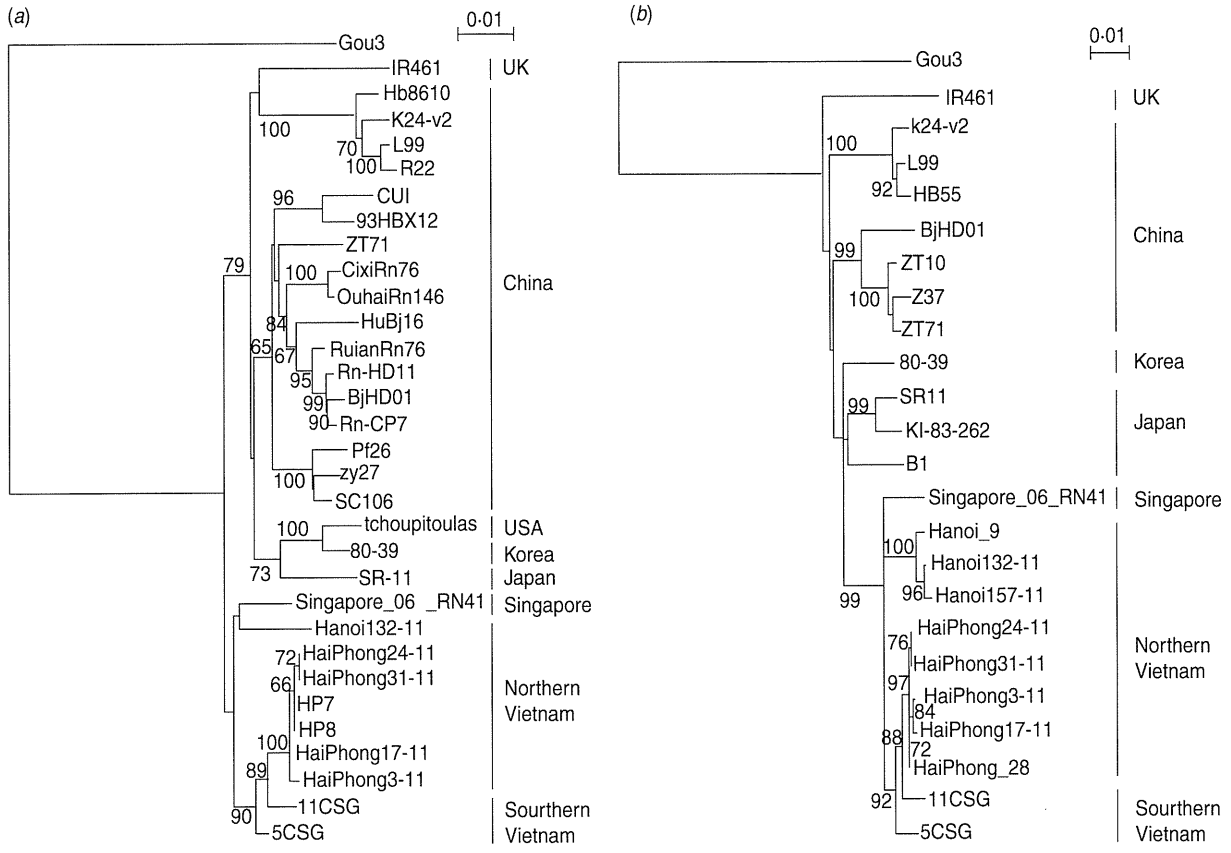

Fig. 2. Phylogenetic analysis of SEOV derived from Hanoi City and the Hai Phong Port area. (*a*) Neighbour Joining (NJ) analysis of hantavirus based on 1378 nt from the S segment. Sequences of SEOV strains Gou3 (AF184988), IR461 (AF329388), Hb8610 (AF288643), K24-v2 (AF288655), L99 (AF288299), R22 (AF288655), CUI (GQ279395), 93HBX12 (EF192308), ZT71 (AY750171), CixiRn76 (FJ803206), OuhaiRn146 (FJ803210), HuBj16 (GQ279380), RuianRn (FJ803216), Rn-HD11 (GQ279392), BjHD01 (AY627049), Rn-CP7 (GQ279383), Pf26 (AY006465), zy27 (AF406965), SC106 (GU361893), tchoupitoulas (AF329389), 80-39 (NC_005236), SR-11 (M34881), Singapore_06_RN41 (GQ274944), 11CSG (AB618113) and 5CSG (AB618112) were used. (*b*) NJ analysis of hantavirus based on 1103 nt from the M segment. Sequences of SEOV strains Gou3 (AF145977), IR461 (AF458104), k24-v2 (AF288654), L99 (AF288298), HB55 (AF035832), BjHD01 (DQ133505), ZT10 (DQ159911), Z37 (AF187081), ZT71 (EF117248), 80-39 (S47716), SR11 (M34882), KI-83-262 (D17594), B1 (AB457794), Singapore_06_RN41 (GQ274942), Hanoi_9 (AB355732), HaiPhong_28 (AB355731), 11CSG (AB618131) and 5CSG (AB618130) were used. Our sequence data for Vietnamese SEOV derived from *R. norvegicus* captured in Hanoi City (S segment; Hanoi132-11) (M segment; Hanoi132-11 and Hanoi157-11) and Hai Phong Port (S segment; HP7, HP8, HaiPhong3-11, HaiPhong17-11, HaiPhong24-11 and HaiPhong31-11) (M segment; HaiPhong3-11, HaiPhong17-11, HaiPhong24-11 and HaiPhong31-11) including our previous data were compared with the published sequence.

populations by horizontal transmission [31, 32]. The lower antibody-positive rates in juvenile *R. norvegicus* might be due to maternal antibodies that prevent vertical transmission [33, 34]. Since the infection rate in sub-adult individuals without maternal antibodies is low, it is speculated that the efficiency of horizontal transmission of the hantavirus is low.

On the other hand, there were juvenile and sub-adult *R. norvegicus* infected with rat HEV. The manner in which rat HEV is transmitted in rats is still unknown. Our data indicated that rat HEV might have vertical transmission in addition to horizontal transmission in rodents. In human cases, vertical transmission of HEV has been reported [35, 36]. In fact, HEV RNA was detected by PCR in cord or birth blood samples of infants born from acute HEV-infected mothers, indicating that HEV is commonly transmitted from infected mothers to their babies [36, 37]. Further experiments on wild rats or laboratory rats are required to demonstrate vertical transmission of rat HEV in rats.

Nevertheless, the density of *R. norvegicus* in Hanoi was higher than that in Hai Phong as indicated by the trapping rate, and the seroprevalence of *L. interrogans*, SEOV and rat HEV in Hanoi was lower than in Hai Phong. It has been reported that the

prevalence of hantavirus in *Peromyscus maniculatus* in North America and that of hantavirus in *Myodes glareolus* in Europe, in which rodents have a seasonal fluctuation of population density, were higher just after the seasonal high population density [38, 39]. However, seasonal patterns in the prevalence of SEOV and *L. interrogans* were not observed in *R. norvegicus* in previous studies [40–42]. Therefore, further longitudinal studies are needed to clarify the relationship between density and *R. norvegicus* seroprevalence in Vietnam.

Our results show that female *R. norvegicus* were more frequently infected with SEOV, *L. interrogans* and rat HEV than males. On the other hand, field studies on SEOV infection in rodents have shown that a higher percentage of infected individuals is commonly observed to be males [43]. Nuttall and Krojgaard *et al.* found no sexual difference in rates of infection with *L. interrogans* in rats [44, 45], whereas Easterbrook *et al.* suggested that female rats are more prone to infection [42]. The reasons for the female-biased SEOV, *L. interrogans* and rat HEV infections are unknown.

Easterbrook *et al.* previously reported that there was a correlation between prevalence of *L. interrogans* infection and HEV infection in *R. norvegicus* but not between SEOV infection and *L. interrogans* or HEV infection [42]. In the present study, no significant correlation was found in any of the pathogens tested. The reasons for no correlation in the pathogens are unknown.

All hantavirus genome-positive specimens were also positive by serological assay in this study. This result provides convincing evidence that animals chronically infected with hantavirus have specific antibodies as reported previously [46].

In our previous phylogenetic study, the M segment of SEOV from Hai Phong formed a distinct clade from those of SEOV from Hanoi [5]. Phylogenetic analyses of the S- and M-segment nucleotide sequences indicated that SEOVs from Hai Phong and Hanoi form different clades. Furthermore, the SEOV from Hai Phong was placed more closely to SEOV from Saigon Port in Ho Chi Minh City (11CSG and 5CSG) compared to SEOV from Hanoi. The cytochrome *b* sequences of *R. norvegicus* in Saigon and some *R. norvegicus* in Hai Phong were identical, but there were small differences between cytochrome *b* sequences of *R. norvegicus* captured in Saigon and Hanoi and between cytochrome *b* sequences of *R. norvegicus* captured in Hanoi and Hai Phong (data not shown). These results indicate that *R. norvegicus* has recently moved between Saigon and Hai Phong. Together with the phylogenetic tree of SEOV, these results suggest that SEOV in Hai Phong might have been transported from Saigon Port with *R. norvegicus*. However, since the distance between Hanoi and Hai Phong is only about 90 km, it is also speculated that variable SEOVs were able to be separately maintained.

Taken together, serological evidence of human pathogens, *L. interrogans*, SEOV and rat HEV, was obtained in *Rattus* spp. captured in urban areas of Northern Vietnam, Hanoi and Hai Phong. Further differential diagnosis of AFI in humans is needed to determine the number of cases of each infection, and continued rodent surveillance is important to estimate the emergence of rodent-borne diseases.

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DECLARATION OF INTEREST

None.

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Development of a serotyping enzyme-linked immunosorbent assay system based on recombinant truncated hantavirus nucleocapsid proteins for New World hantavirus infection

Takaaki Koma[a], Kumiko Yoshimatsu[a], Midori Taruishi[a,1], Daisuke Miyashita[b], Rika Endo[a], Kenta Shimizu[a], Shumpei P. Yasuda[a], Takako Amada[a], Takahiro Seto[b], Ryo Murata[b], Haruka Yoshida[b], Hiroaki Kariwa[b], Ikuo Takashima[b], Jiro Arikawa[a,*]

[a] *Department of Microbiology, Graduate School of Medicine, Hokkaido University, Kita-ku, Kita-15, Nishi-7, Sapporo 060-8638, Japan*
[b] *Laboratory of Public Health, Graduate School of Veterinary Medicine, Hokkaido University, Kita-ku, Kita-18, Nishi 9, Sapporo 060-0818, Japan*

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A B S T R A C T

New World hantaviruses were divided into five groups based on the amino acid sequence variability of the internal variable region (around 230–302 amino acids) of hantavirus nucleocapsid protein (NP). Sin Nombre virus (SNV), Andes virus, Black Creek Canal virus (BCCV), Carrizal virus (CARV) and Cano Delgadito virus belong to groups 1, 2, 3, 4 and 5, respectively. Patient and rodent sera were serotyped successfully by an enzyme-linked immunosorbent assay (ELISA) with recombinant truncated NP lacking 99 N-terminal amino acids (trNP100) of SNV, CARV and BCCV. The trNP100 of BCCV showed lower reactivity to heterologous sera. In contrast, whole recombinant NP antigens detected both homologous and heterologous antibodies equally. The results together with results of a previous study suggest that trNP100 can distinguish infections among viruses in groups 1, 2, 3 and 4 of New World hantaviruses. The serotyping ELISA with trNP100 is useful for epidemiological surveillance in humans and rodents.

1. Introduction

Hantaviruses belong to the family *Bunyaviridae* and are maintained in rodents and other small mammals that are infected persistently (Schmaljohn and Hjelle, 1997). Hantaviruses cause two febrile illnesses in humans, hemorrhagic fever with renal syndrome (HFRS) in the Old World and hantavirus pulmonary syndrome (HPS) in the New World (Kariwa et al., 2007; Schmaljohn and Hjelle, 1997). Transmission of the viruses to humans occurs through inhalation of aerosolized animal excreta or rodent bites (Lee and van der Groen, 1989; Meyer and Schmaljohn, 2000). Hantaviruses appear to have co-evolved with the rodent reservoir host species over many thousands of years (Hughes and Friedman, 2000; Schmaljohn and Hjelle, 1997). The difference in epidemic areas of HFRS and HPS depends on the rodent habitat (Zeier et al., 2005).

Hantavirus virions contain three segmented negative-sense RNAs designated S, M, L; they encode a nucleocapsid protein (NP), enveloped glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase (L protein), respectively (Elliott, 1990; Schmaljohn, 1996). Hantavirus NP is the most abundant viral component in both virions and infected cells and can form a stable trimer (Elliott et al., 2000; Kaukinen et al., 2001, 2004). The NP of hantaviruses possesses immunodominant, linear, cross-reactive epitopes around the first 100 amino acids (aa) of the N-terminus (Elgh et al., 1996; Gott et al., 1997; Vapalahti et al., 1995; Yamada et al., 1995). On the other hand, the variable region around 230–302 aa forms serotype-specific epitopes after multimerization of NP (Tischler et al., 2008; Yoshimatsu et al., 2003).

Recombinant antigens were expressed with multimerization-dependent serotype-specific epitopes after truncation of the N-terminal 49 aa in NP (trNP50) by a baculovirus (Araki et al., 2001; Nakamura et al., 2008; Yasuda et al., 2012). Enzyme-linked immunosorbent assay (ELISA) using trNP50 differentiated successfully infections with four different serotypes of Old World hantavirus: Hantaan, Seoul, Dobrava, and Thailand viruses in HFRS patient and rodent sera (Araki et al., 2001; Nakamura et al., 2008). ELISA using trNP lacking 99 aa of the N-terminal end of the NP (trNP100) differentiated successfully infections with three different serotypes of New World hantaviruses: Sin Nombre virus (SNV), Andes virus (ANDV) and Laguna Negra virus (LANV) in HPS patient and rodent sera (Koma et al., 2010). Therefore, the serotyping ELISA using trNPs is a more rapid, safe and simple method as a substitute

* Corresponding author. Tel.: +81 11 706 6905; fax: +81 11 706 7879.
E-mail address: j_arika@med.hokudai.ac.jp (J. Arikawa).
[1] Present address: Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.

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for the neutralization test, which has been the only serological assay for determining the serotype (Araki et al., 2001; Koma et al., 2010; Nakamura et al., 2008).

Since the first recognition of HPS in the United States in 1993, more than 30 new hantaviral strains or genetic lineages have been identified from patients with HPS or various rodent species throughout the Americas (Jonsson et al., 2010; Peters and Khan, 2002; Schmaljohn and Hjelle, 1997). However, the antigenic relationship among the New World hantaviruses has not been studied in detail.

Since serotyping with trNP depended on the antigenic difference of serotype-specific epitopes within the internal region of trNP, it was expected that variability of the aa sequences in the region also correlated to the hantavirus serotype. In the present study, therefore, amino acid sequences of the internal variable regions of NP of many New World hantaviruses were compared. The results showed that they were divided into 5 groups. Therefore, SNV (group 1) and Black Creek Canal virus (BCCV) (group 3), which were associated with HPS in the United States (Hjelle et al., 1994; Ravkov et al., 1995), and Carrizal virus (CARV) (group 4), which was recognized recently as a New World hantavirus isolated from *Reithrodontomys sumichrasti* in Mexico (Kariwa et al., 2012), were selected, and the applicability of their trNPs for a serotyping antigen was examined.

2. Materials and methods

2.1. cDNAs

cDNAs containing coding information for the S segment of SNV strain SN 77734 (Botten et al., 2000), CARV strain 2/2006 (Kariwa et al., 2012) and BCCV (GenBank ID: AB689163) were used. CARV was recognized recently from *R. sumichrasti* in Mexico (Kariwa et al., 2012).

2.2. Monoclonal antibodies and human and rodent sera

Monoclonal antibodies (MAbs) to the NP of HTNV and PUUV were used for antigenic characterization of the NP by an indirect immunofluorescence assay (IFA). The MAbs 2E12, 4C3, 4E5, GBO4, ECO2 and ECO1 recognize the N-terminal epitope of the NP. The MAbs F23A1 and E5/G6 recognize aa 291–402 and aa 165–173 of the NP, respectively (Lundkvist et al., 1991; Ruo et al., 1991; Yoshimatsu et al., 1996). The epitope for MAb C16D11 is unknown. MAbs except for GBO4 and ECO1 were obtained from the cell culture supernatant. The MAbs GBO4 and ECO1 were obtained from ascitic fluid. Sera from three patients infected with SNV were supplied kindly by Brian Hjelle of the University of New Mexico Health Sciences Center, New Mexico, USA. Negative control human sera were obtained from healthy volunteers. This study was approved by the ethics committee of Hokkaido University Graduate School of Medicine, and informed consent was obtained from all human subjects, including healthy volunteers. Three sera from *Peromyscus maniculatus* infected with SNV and one serum from hantavirus-uninfected *P. maniculatus* were supplied kindly by David Safronetz of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Montana, USA. Several species of *Reithrodontomys* were captured in Guerrero, Mexico. Three *R. sumichrasti* were infected with CARV, and one *R. megalotis* was infected with Huitzilac virus (HUIV), which showed 96.7% amino acid identity to NP of CARV (Kariwa et al., 2012). Sera from hantavirus-uninfected *P. maniculatus*, *R. sumichrasti* and *R. megalotis* were used as negative controls. These viral types in the patients and rodents were determined by detection of the virus genome by reverse transcriptase (RT)-PCR.

2.3. Amino acid and nucleotide sequence comparison and phylogenetic analysis

Amino acid and nucleotide sequences of the variable region in NP (230–302 aa and 690–906 nucleotides) of New World hantavirus in North America and South America were aligned and compared with sequences determined previously using GenetyxMac Ver.13 (Genetyx Corporation, Tokyo, Japan). Phylogenetic relationships among the hantavirus sequences of the variable region of NP were evaluated using the Neighbor-Joining program with the Kimura 2 parameter distance in CLUSTALW version 1.83 (European Bioinformatics Institute, Cambridge, UK). The phylogenetic tree was visualized using the NJ plot program (Perriere and Gouy, 1996). Bootstrap resampling analysis was performed using 1000 replicates.

2.4. Construction of recombinant baculoviruses expressing whole rNPs and trNPs

The gene encoding whole NP (aa 1–428) and truncated genes encoding truncated NP (aa 50–428 and aa 100–428) were PCR-amplified from cDNA of the S segment. The primers listed below were used for amplification of whole and truncated S segments. A 5′ SpeI site and a 3′ XhoI site were introduced into the primers (both sites shown in italics). Primer sequences (forward and reverse) were as follows: SNV whole rNP, 5′-gac*actagt*atgagcaccctcaaagaa-3′ and 5′-tac*ctcgag*ttaaagtttaagtttaagtggttc-3′; CARV whole rNP, 5′-aaa*actagt*atgagcaacctcaaagaa-3′ and 5′-gat*ctcgag*ttatagttttagagg-3′; BCCV whole rNP, 5′-gaa*actagt*atgagcaacctcaaagaa-3′ and 5′-gatt*ctcgag*tcatatctttaaaggctc-3′; SNV trNP50, 5′-tcg*actagt***atg**gctgtgtctgcattggag-3′ and 5′-tac*ctcgag*ttaaagtttaagtttaagtggttc-3′; CARV trNP50, 5′-aga*actagt***atg**gctgtgtctgcattggaa-3′ and 5′-gat*ctcgag*ttatagttttagagg-3′; BCCV trNP50, 5′-aac*actagt***atg**gctgtgtctgcattggag-3′ and 5′-gatt*ctcgag*tcatatctttaaaggctc-3′; SNV trNP100, 5′-tcg*actagt***atg**gctgtgtctgcattggag-3′ and 5′-tac*ctcgag*ttaaagtttaagtttaagtggttc-3′; CARV trNP100, 5′-aga*actagt***atg**gctgtgtctgcattggaa-3′ and 5′-gat*ctcgag*ttatagttttagagg-3′; BCCV trNP100, 5′-ctt*actagt***atg**aatgtgcttgacgtcaat-3′ and 5′-gatt*ctcgag*tcatatctttaaaggctc-3′. Boldface indicates an added start codon. After amplification, the DNA fractions were subcloned into pFastBac1 (Invitrogen, Groningen, The Netherlands). The recombinant baculoviruses were expressed using the Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer's instructions. Mock baculovirus was made from original pFastBac1. The titers of recombinant baculoviruses in the culture supernatant were determined by 50% tissue culture infective dose ($TCID_{50}$) with High Five cells.

2.5. Preparation of whole rNPs and trNPs expressed by baculoviruses

High Five cells (Invitrogen) were grown in Grace's insect cell culture medium (Invitrogen) supplemented with 10% fetal bovine serum as described previously (Araki et al., 2001). High Five cells were infected with recombinant baculoviruses at a multiplicity of infection of 1 for 3 days. Collection and lysis of infected cells were performed using methods described previously (Araki et al., 2001). Briefly, infected High Five cells were collected in phosphate-buffered saline (PBS) of 2.5×10^6 cells/mL and sonicated. The cell lysate containing recombinant NP (rNP) was used as ELISA antigen. The lysate of cells infected with mock baculovirus was used as a negative control. The expression of rNPs of SNV, CARV and BCCV was confirmed by Western blotting (data not shown) using methods described previously (Yoshimatsu et al., 1995). High Five cells expressing whole recombinant NPs (whole rNPs) of PUUV

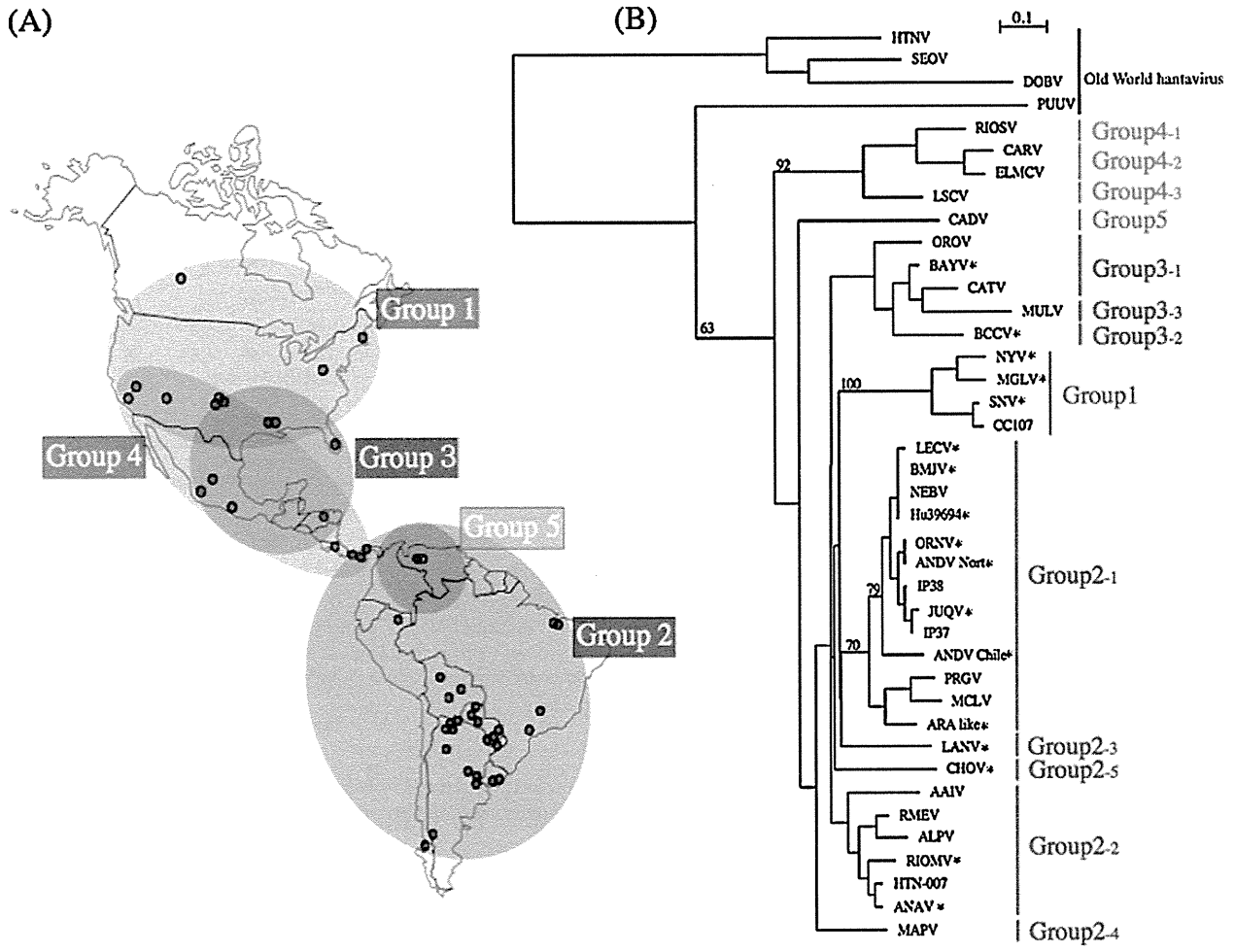

Fig. 1. Distribution map of grouped New World hantaviruses and phylogenetic tree for New World hantavirus. (A) The map represents the geographical distribution of grouped New World hantaviruses. (B) Phylogenetic tree for New World hantavirus. Neighbor-joining phylogenetic analysis was performed on the basis of partial aa sequences of S (aa 230–302). An asterisk (*) indicates that human cases of infection with the virus have been reported. Abbreviations: AAIV, Ape Aime Itapua virus, Hantavirus strain IP16 (GenBank ID: DQ345764); ALPV, Alto Paraguay virus (GenBank ID: DQ345762); ANAV, Anajatuba virus (GenBank ID: DQ451829); ANDV Chile, Andes virus Chile-9717869 (GenBank ID: AF291702); ANDV Nort, Andes virus AND Nort (GenBank ID: AF325966); ARA like, Araraquara-like virus strain P5/Cajuru (GenBank ID: EF571895); BAYV, Bayou virus (GenBank ID: L36929); BCCV, Black Creek Canal virus (GenBank ID: L39949); BMJV, Bermejo virus (GenBank ID: AF482713); CARV, Carrizal virus (GenBank ID: AB620093); CATV, Catacamas virus (GenBank ID: DQ256126); CC107, Convict Creek 107 virus (GenBank ID: L33683); CADV, Cano Delgadito virus (GenBank ID: DQ285566); CHOV, Choclo virus (GenBank ID: DQ285046); DOBV, Dobrava-Belgrade virus (GenBank ID: L41916); ELMCV, El Moro Canyon virus (GenBank ID: U11427); HTNV, Hantaan virus (GenBank ID: M14626); HTN-007, Hantavirus HTN-007 (GenBank ID: AF133254); Hu39694, Hu39694, Hantavirus sp. (GenBank ID: AF482711); IP37, Hantavirus strain Itapua 37 (GenBank ID: DQ345765); IP38, Hantavirus strain Itapua 38 (GenBank ID: DQ345766); JUQV, Juquitiba virus (GenBank ID: EF492472); LANV, Laguna Negra virus (GenBank ID: AF005727); LECV, Lechiguanas virus (GenBank ID: AF482714); LSCV, Limestone Canyon virus (GenBank ID: AF307322); MCLV, Maciel virus (GenBank ID: AF482716); MAPV, Maporal virus (GenBank ID: AY267347); MGLV, Hantavirus Monongahela-1 (GenBank ID: U32591); MULV, Muleshoe virus (GenBank ID: U54575); NEBV, Neembucu hantavirus (GenBank ID: DQ345763); NYV, New York virus (GenBank ID: U09488); ORNV, Oran virus (GenBank ID: AF482715); OROV, Playa de Oro hantavirus (GenBank ID: EF534079); PRGV, Pergamino virus (GenBank ID: AF482717); PUUV, Puumala virus (GenBank ID: X61035); RIOMV, Rio Mamore virus (GenBank ID: U52136); RMEV, Rio Mearim virus (GenBank ID: DQ451828); RIOSV, Rio Segundo virus (GenBank ID: U18100); SEOV, Seoul, Sapporo rat virus (GenBank ID: M34881); SNV, Sin Nombre virus SN 77734 (GenBank ID: AF281851).

and HTNV were prepared as described previously (Araki et al., 2001). High Five cells expressing whole rNPs and trNPs were used for IFA.

2.6. Preparation of rNPs expressed by Escherichia coli

Whole rNPs of SNV, CARV and BCCV fused with a Nus-tag and His-tag were expressed in *E. coli*. DNA fractions containing the entire coding region of NP of SNV, CARV and BCCV were made by digestion of pFastBac1 including the cDNA with SalI 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, USA) containing ampicillin (50 μg/mL) for small-scale culture incubation at 37 °C overnight. After the culture fluid had been 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 four times for 15 s each time on ice. Thereafter, the fusion protein was purified using a His-Trap HP (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. An antigen made from the original pET43b vector was used as a negative control. The purities of recombinant antigen were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown).

2.7. IFA test

Acetone-fixed smears of High Five cells infected with recombinant baculoviruses were used as antigens. The detailed procedure was described previously (Morii et al., 1998; Yoshimatsu et al., 1993). MAbs obtained from the cell culture supernatant were used without dilution. MAbs GBO4 and ECO1 obtained from ascitic fluid were used at 100-fold dilution.

2.8. Detection of multimerized rNPs

To detect multimerization of the rNPs expressed by the baculovirus, competitive-sandwich ELISA was performed with MAb E5/G6 recognizing aa 165–173 as a capture and detector antibody. Briefly, rNPs were captured on the plate with MAb E5/G6 followed by detection with the same MAb E5/G6. Positive reaction with this ELISA indicates that the antigens are forming a multimer (Yoshimatsu et al., 2003).

2.9. ELISA with whole rNPs expressed by E. coli

ELISA using whole rNPs expressed by *E. coli* was carried out as described previously (Koma et al., 2010). Patient and rodent sera were used at 200-fold dilution. Horseradish peroxidase (HRP)-labeled goat anti-human IgG (H + L) antibody (KPL, Gaithersburg, MD, USA) for patient sera and HRP-labeled goat anti-*Peromyscus leucopus* IgG (H + L) antibody (KPL) for *Peromyscus* and *Reithrodontomys* sera were used as secondary antibodies. Color reactions were performed with *o*-phenylenediamine dihydrochloride (OPD) (Sigma–Aldrich, St. Louis, MO) and allowed to develop for 10–15 min. Absorbance was measured at 450 nm by using a SpectraMax 340 microplate spectrophotometer (Molecular Device, Sunnyvale, CA). An antigen made from the original pET43b vector was used as a negative control.

2.10. Serotyping ELISA with trNPs expressed by baculovirus

The serotyping ELISA was performed as described previously (Koma et al., 2010; Nakamura et al., 2008). Ninety-six-well plates were coated with MAb E5/G6 (2 μg/mL in PBS) as a capture antibody. Patient and rodent sera were used at 200-fold dilution. HRP-labeled goat anti-human IgG (H + L) antibody (KPL) for patient sera and HRP-labeled goat anti-*Peromyscus leucopus* IgG (H + L) antibody (KPL) for *Peromyscus* and *Reithrodontomys* sera were used as secondary antibodies. Color development results were the same as those for the ELISA with whole rNPs. Cell lysate infected with mock baculovirus was used as a negative control.

3. Results

3.1. Grouping of New World hantaviruses by comparison of the variable region of NP

New World hantaviruses were divided into five groups (groups 1–5) based on the identity of aa in the internal variable region (aa 230–302) (Table 1). Groups were defined as more than approximately 70% amino acid sequence identity except for Bayou virus (BAYV) and Playa de Oro virus (OROV) in group 3. These two hantaviruses have more than 70% amino acid sequence identity to those of most of the viruses in group 2, but they have higher amino acid sequence identity to those of viruses in group 3. Subgroups were defined as more than approximately 80% of amino acid identity. Based on this classification, groups 2, 3 and 4 were divided into five, three and three subgroups, respectively. This classification corresponded to geographical characteristics and clades of the phylogenetic tree of the virus (Fig. 1). The endemic areas of groups 1, 3 and 4 were overlapped in the southern area of the United States. In this study, SNV, BCCV and CARV were selected as representative viruses of groups 1, 3 and 4, respectively. As shown in Table 1, aa sequence identities among SNV, BCCV and CARV ranged from 49.3% to 54.8%.

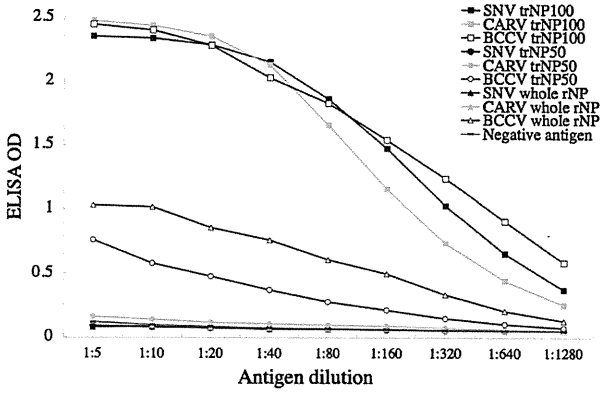

Fig. 2. Multimerization of rNPs in competitive-sandwich ELISA. Antigens were captured and detected with MAb E5/G6. Each antigen was diluted from 1:5 to 1:1280 and subjected to capture ELISA. Positive reaction with this ELISA indicates that the antigens are forming a multimer. The ELISA was performed three times and the representative OD value was plotted.

3.2. Antigenic characterization of rNPs expressed by recombinant baculovirus with MAbs in IFA tests

Antigenic profiling of whole rNPs or trNPs of SNV, CARV and BCCV expressed in High Five cells was carried out using hantavirus-specific MAbs (Table 2). Whole rNPs of SNV, CARV and BCCV reacted to cross-reactive MAbs (2E12, 4C3, 4E5, GBO4, C16D11, ECO2 and ECO1) that recognized immunodominant epitopes of the N-terminus of NP, except for C16D11, and cross-reactive MAbs (F23A1 and E5/G6) that recognized the C-terminus of NP aa 291–402 and aa 166–175, respectively. The reactivity pattern of the SNV whole rNP was different from those of the whole rNPs of CARV and BCCV with MAbs C16D11 and ECO2. The trNP50s of SNV, CARV and BCCV lacked reactivity with 5 of the N-terminal specific MAbs (2E12, 4C3, 4E5, ECO2 and ECO1) but still reacted with MAb GBO4, which recognized the N-terminus. The trNP100s reacted to only two cross-reactive MAbs, E5/G6 and F23A1.

3.3. Detection of multimerization of rNPs

Multimerization activities of whole rNPs, trNP50s and trNP100s were compared among those from SNV, CARV and BCCV. As shown in Fig. 2, there was no reaction to trNP50s and whole rNPs of SNV and CARV. This implied that the trNP50s and whole rNPs captured by E5/G6 could not react with E5/G6 as a detector due to competition. Thus, trNP50s and whole rNPs of SNV and CARV were considered as monomers. On the other hand, there were strong reactions to trNP100s, indicating that trNP100s of SNV, CARV and BCCV existed as multimers. There were moderate reactions to whole rNP and trNP50 of BCCV. Since serotype-specific epitopes have been suggested to be formed after multimerization of trNPs (Yoshimatsu et al., 2003), trNP100s were selected as ELISA antigens for serotyping ELISA. The reactivities of trNP100s of SNV, CARV and BCCV were nearly equivalent at antigen dilutions of 1:5 to 1:40. Therefore, the antigens were used at 10-fold dilution.

Table 1
Identity of amino acid (upper right) and nucleotide (lower left) sequences of the S segment of hantaviruses.

	Group 1			Group 2-1								2-2				2-3	2-4	2-5	Group 3-1			3-2	3-3	Group 4-1	4-2		4-3	Group 5	Old World hantavirus			
Virus	SN	NY	MGL	Hu 39694	LEC	ORN	JUQ	AND Chile	ARA like	PRG	MCL	ANA	RIOM	ALP	AAI	LAN	MAP	CHO	BAY	ORO	CAT	BCC	MUL	RIOS	ELMC	CAR	LSC	CAD	PUU	HTN	SEO	DOB
SN		**84.9**	**78.1**	**69.9**	**68.5**	**67.1**	**67.1**	**61.6**	**67.1**	**63.0**	**61.6**	**64.4**	**61.6**	**64.4**	**65.8**	**63.0**	**60.3**	**61.6**	**60.3**	**61.6**	**60.3**	**53.4**	**52.1**	**57.5**	**56.2**	**54.8**	**54.8**	**57.5**	**39.7**	**28.8**	**26.0**	**23.3**
NY	82.6		89.0	65.8	64.4	63.0	63.0	61.6	64.6	63.0	60.3	65.8	63.0	64.4	65.8	64.4	60.3	61.6	61.6	63.0	63.0	**56.2**	56.2	53.4	53.4	**52.1**	54.8	56.2	35.6	30.1	26.0	24.7
MGL	74.9	82.2		68.5	67.1	65.8	63.0	64.4	64.4	64.4	61.6	64.6	61.6	63.0	67.1	63.0	60.3	61.6	61.6	60.3	61.6	**67.5**	53.4	49.3	49.3	**47.9**	52.1	54.8	37.0	28.8	26.0	24.7
Hu39694	65.3	63.5	68.8		98.6	95.9	91.8	89.0	86.3	82.2	80.8	76.7	76.7	74.0	72.6	74.0	72.6	71.2	74.0	72.6	65.8	**68.5**	60.3	50.7	49.3	**49.3**	56.2	63.0	41.1	32.9	27.4	27.4
LEC	68.5	63.5	66.4	81.3		94.5	90.4	87.7	84.9	80.8	79.5	75.3	78.1	72.6	71.2	72.6	74.0	69.9	72.6	71.2	64.4	**67.1**	58.9	52.1	47.9	**47.9**	54.8	64.4	39.7	34.2	28.8	28.8
ORN	68.0	67.0	68.7	80.4	85.4		94.5	86.3	83.6	80.8	79.5	75.3	75.3	72.6	71.2	72.6	71.2	68.5	72.6	71.2	64.4	**67.1**	58.9	50.7	49.3	**49.3**	56.2	60.3	39.7	32.9	27.4	29.2
JUQ	67.6	66.7	64.2	78.1	78.5	78.1		87.7	82.2	78.1	79.5	78.1	74.0	71.2	69.9	75.3	71.2	71.2	71.2	72.6	65.8	**64.4**	57.5	50.7	50.7	**49.3**	57.5	60.3	39.7	31.5	28.8	27.4
AND Chile	64.8	68.3	66.5	75.8	76.3	74.4	73.5		79.5	80.8	78.1	75.3	72.6	67.1	68.5	71.2	72.6	69.9	71.2	68.5	64.4	**67.1**	61.6	49.3	46.6	**45.2**	58.9	60.3	39.7	30.1	30.1	28.8
ARA like	70.3	63.0	64.8	76.7	77.2	75.8	70.3	73.1		84.9	83.6	78.1	72.6	78.1	71.2	71.2	74.0	71.2	74.0	74.0	68.5	**64.4**	65.8	52.1	53.4	**53.4**	56.2	60.3	43.8	32.9	27.4	28.8
PRG	67.1	66.7	68.8	74.9	74.4	74.4	74.4	73.5	76.7		89.0	76.7	74.0	75.3	67.1	69.9	72.6	68.5	71.2	74.0	65.8	**65.8**	61.6	52.1	49.3	**50.7**	61.6	56.2	43.8	28.8	27.4	30.5
MCL	65.6	63.3	61.5	73.5	78.1	77.2	73.1	73.5	74.4	77.2		76.7	74.0	76.7	68.5	65.8	71.2	65.8	69.9	72.6	65.8	**65.8**	61.6	52.1	52.1	**49.3**	61.6	56.2	46.6	28.8	28.8	27.4
ANA	67.6	63.0	61.6	71.2	74.0	71.7	73.1	67.1	72.1	68.0	68.5		90.4	89.0	78.1	78.1	79.5	75.3	72.6	74.0	71.2	**67.1**	61.6	54.8	52.1	**53.4**	64.4	61.6	38.4	32.9	30.1	26.0
RIOM	64.8	67.9	66.1	68.9	73.1	71.7	69.9	68.0	69.9	70.3	66.2	81.7		82.2	78.1	75.3	78.1	69.9	69.9	69.9	67.1	**67.1**	58.9	56.2	50.7	**52.1**	63.0	60.3	37.0	35.6	34.2	27.4
ALP	66.2	66.5	67.4	70.3	70.8	70.3	67.1	64.8	69.9	68.9	67.6	78.5	74.4		78.1	74.0	71.2	65.8	69.9	68.5	67.1	**65.8**	60.3	56.2	56.2	**56.2**	60.3	64.4	41.1	28.8	27.4	26.0
AAI	67.6	66.2	63.0	68.0	66.7	68.0	70.8	64.4	66.7	69.9	68.0	72.6	72.6	73.1		68.5	71.2	68.5	74.0	71.2	68.5	**65.8**	61.6	54.8	52.1	**52.1**	57.5	60.3	35.6	32.9	27.4	24.7
LAN	62.6	63.9	63.8	71.7	66.7	68.9	74.4	68.9	68.9	69.9	63.0	71.2	74.9	67.6	67.6		71.2	67.1	64.4	68.5	64.4	**60.3**	54.8	54.8	54.8	**56.2**	56.2	60.3	35.6	34.2	27.4	26.0
MAP	63.0	62.6	63.6	67.1	68.0	67.1	67.6	67.1	67.1	69.4	67.1	70.3	74.9	68.0	72.1	68.5		68.5	74.0	69.9	69.9	**65.8**	61.6	56.2	50.7	**52.1**	61.6	61.6	37.0	38.4	34.2	32.9
CHO	60.3	58.9	61.0	69.4	66.7	65.3	65.8	66.2	65.3	66.2	66.7	68.0	65.8	64.8	66.2	66.2	63.0		69.9	67.1	64.4	**63.0**	58.9	53.4	53.4	**53.4**	60.3	58.9	38.4	30.1	27.4	26.0
BAY	63.9	66.7	60.7	63.5	66.2	67.1	67.6	63.5	68.0	66.2	63.5	68.9	68.5	65.8	70.8	66.7	67.1	64.2		86.3	87.7	**82.2**	80.8	58.9	57.5	**54.8**	60.3	54.8	39.7	31.5	27.4	27.4
ORO	63.9	62.6	61.6	69.9	68.9	68.5	71.2	63.9	68.5	69.4	65.3	69.4	68.0	65.8	70.8	68.9	68.0	66.7	75.8		82.2	**72.6**	74.0	58.9	54.8	**53.4**	58.9	53.4	41.1	30.1	27.4	31.5
CAT	62.1	61.2	59.4	61.6	64.4	63.0	65.8	64.8	64.8	66.7	62.6	65.8	65.8	66.7	67.6	66.2	67.1	63.9	78.5	76.3		**74.0**	78.1	58.9	56.2	**53.4**	56.2	52.1	39.7	32.9	26.0	27.4
BCC	63.3	62.4	61.0	63.5	68.9	65.3	68.5	64.8	65.3	66.2	64.8	68.9	69.9	68.9	67.1	66.7	68.9	66.7	75.3	74.0	75.8		**71.2**	**53.4**	**50.7**	**49.3**	**57.5**	**52.1**	**46.6**	**30.1**	**28.8**	**27.4**
MUL	62.1	60.3	58.9	62.6	65.4	65.0	65.1	64.1	61.6	67.3	64.7	67.6	64.4	65.1	66.2	65.1	65.8	62.6	73.5	70.8	74.4	76.1		50.7	49.3	**46.6**	52.1	49.3	43.8	27.8	24.7	27.4
RIOS	60.3	57.5	57.1	61.6	60.6	63.3	61.2	60.3	61.2	62.4	58.3	59.4	61.6	63.0	61.6	60.3	63.0	63.9	62.1	64.4	61.5	61.5	60.6		78.1	**78.1**	69.9	53.4	34.2	28.8	27.4	28.8
ELMC	58.4	56.6	60.4	58.4	58.0	60.6	60.3	63.1	62.8	60.1	58.0	59.2	59.2	58.7	59.8	57.3	61.6	57.8	59.8	63.5	62.8	63.0	56.2	74.0		**90.4**	74.0	54.8	32.9	27.4	27.4	26.0
CAR	64.4	61.1	59.9	56.2	58.0	61.3	57.1	54.8	58.7	59.2	63.1	61.5	58.5	61.8	63.1	60.4	57.1	63.1	61.2	61.5	60.4	61.0	62.7	68.5	74.9		**69.9**	**53.4**	**32.9**	**30.1**	**23.3**	**26.0**
LSC	61.2	56.6	55.3	62.6	62.8	63.8	60.3	60.3	63.0	62.1	61.6	64.2	63.3	61.6	64.8	55.7	63.0	61.6	59.4	65.8	58.4	64.4	60.3	68.9	71.7	68.0		52.1	34.2	30.1	32.9	28.8
CAD	63.6	63.3	58.9	66.1	71.4	66.1	71.9	65.6	65.6	64.2	64.8	66.5	60.7	65.6	64.8	64.2	67.0	61.9	65.1	60.3	58.9	61.9	59.8	65.0	66.4	62.4	63.1		35.6	31.5	24.7	26.0
PUU	53.9	54.4	56.0	56.4	53.0	58.7	61.8	58.0	53.9	59.2	58.1	51.6	53.7	57.8	56.6	59.3	56.9	54.8	53.9	55.8	56.6	56.7	54.2	55.0	57.1	60.5	51.1	51.9		24.3	27.0	28.4
HNT	55.2	54.6	52.1	53.7	52.6	50.9	61.6	54.2	52.8	55.6	52.3	53.5	60.3	48.4	52.3	56.9	55.9	53.2	53.2	53.5	53.2	55.4	56.9	51.9	50.2	48.4	56.9	55.7	53.3		54.1	58.1
SEO	47.5	52.9	48.2	55.3	56.5	54.9	48.2	53.5	50.2	53.3	45.0	48.4	47.0	50.7	46.5	49.7	53.7	47.5	47.2	48.1	51.0	50.9	47.5	54.9	52.1	50.6	47.5	49.1	47.9	61.2		56.8
DOB	53.5	50.0	53.8	52.1	54.0	52.7	51.5	51.2	49.8	51.2	49.8	53.7	53.5	52.9	51.6	55.0	53.1	54.7	50.7	56.1	46.5	50.9	48.8	46.8	51.6	54.9	54.9	51.9	53.4	59.5	64.3	

Amino acid and nucleotide identities of variable region (230–302 amino acids and 690–906 nucleotides). Abbreviations: AAI, Ape Aime Itapua virus, Hantavirus strain IP16 (GenBank ID: DQ345764); ALP, Alto Paraguay virus (GenBank ID: DQ345762); ANA, Anajatuba virus (GenBank ID: DQ451829); AND Chile, Andes virus Chile-9717869 (GenBank ID: AF291702); ARA like, Araraquara-like virus strain P5/Cajuru (GenBank ID: EF571895); BAY, Bayou virus (GenBank ID: L36929); BCC, Black Creek Canal virus (GenBank ID: L39949); CAR, Carrizal virus (GenBank ID: AB620093); CAT, Catacamas virus (GenBank ID: DQ256126); CAD, Cano Delgadito virus (GenBank ID: DQ285566); CHO, Choclo virus (GenBank ID: DQ285046); DOB, Dobrava-Belgrade virus (GenBank ID: L41916); ELMC, El Moro Canyon virus (GenBank ID: U11427); HTN, Hantaan virus (GenBank ID: M14626); Hu39694, Hu39694, Hantavirus sp. (GenBank ID: AF482711); JUQ, Juquitiba virus (GenBank ID: EF492472); LEC, Lechiguanas virus (GenBank ID: AF482714); LAN, Laguna Negra virus (GenBank ID: AF005727); LSC, Limestone Canyon virus (GenBank ID: AF307322); MCL, Maciel virus (GenBank ID: AF482716); MAP, Maporal virus (GenBank ID: AY267347); MGL, Hantavirus Monongahela-1 (GenBank ID: U32591); MUL, Muleshoe virus (GenBank ID: U54575); NY, New York virus (GenBank ID: U09488); ORN, Oran virus (GenBank ID: AF482715); ORO, Playa de Oro hantavirus (GenBank ID: EF534079); PRG, Pergamino virus (GenBank ID: AF482717); PUU, Puumala virus (GenBank ID: X61035); RIOM, Rio Mamore virus (GenBank ID: U52136); RIOS, Rio Segundo virus (GenBank ID: U18100); SEO, Seoul, Sapporo rat virus (GenBank ID: M34881); SN, Sin Nombre virus SN 77734 (GenBank ID: AF281851).

Table 2
Antigenic characterization of rNPs expressed by recombinant baculovirus with MAbs in the IFA test.

Origin	MAbs	Epitope	Whole rNP						trNP50			trNP100		
			PUUV[a]	HTNV[a]	SEOV[a]	SNV[a]	CARV[a]	BCCV[a]	SNV	CARV	BCCV	SNV	CARV	BCCV
PUUV	2E12	N-terminus	+[b]	±	±	+	+	+	–	–	–	–	–	–
	4C3	N-terminus	+	+	+	+	+	+	–	–	–	–	–	–
	4E5	N-terminus	+	+	±	+	+	+	–	–	–	–	–	–
	GBO4[c]	N-terminus	+	+	+	+	+	+	±	+	±	–	–	–
HTNV	C16D11	Unknown	+	+	+	–	+	+	–	+	+	–	–	–
	ECO2	aa 1–33	–	+	+	–	+	+	–	–	–	–	–	–
	ECO1[c]	aa 34–103	+	+	+	+	+	+	–	–	–	–	–	–
	F23A1	aa 291–402	–	+	+	+	+	+	+	±	+	+	±	+
	E5/G6	aa 165–173	+	+	+	+	+	+	+	+	+	+	+	+

[a] PUUV, Puumala virus; HTNV, Hantaan virus; SEOV, Seoul virus; SNV, Sin Nombre virus; CARV, Carrizal virus; BCCV, Black Creek Canal 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.

3.4. Reactivities of whole rNP and trNP100s with infected patient and rodent sera

Applicability of the trNP100s in ELISA was examined using patient and rodent sera. As shown in Fig. 3A, whole rNPs of SNV, CARV and BCCV expressed by *E. coli* showed strong cross-reactivity, but trNP100s of SNV and CARV expressed by baculoviruses showed strong reactions to homologous antigen and weak reactions to heterologous antigen (Fig. 3B). The ELISA ODs for the homologous reaction were more than twice those for the heterologous reactions. There was no serum from a BCCV-infected patient or rodent, but the trNP100 of BCCV also showed weak reactivity to heterologous sera.

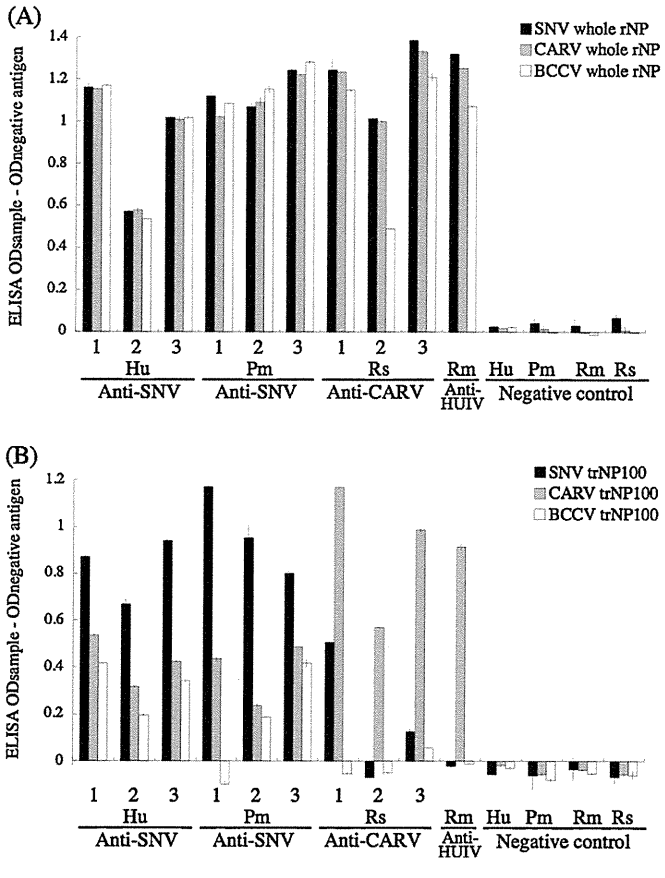

Fig. 3. Reaction patterns of hantavirus-positive and -negative sera in ELISA. (A) Reaction patterns of whole rNPs (SNV, CARV and BCCV) with human and rodent sera in ELISA. (B) Reaction patterns of trNP100s (SNV, CARV and BCCV) with human and rodent sera in ELISA. Abbreviations: Hu, human serum; Pm, *Peromyscus maniculatus* serum; Rs, *Reithrodontomys sumichrasti* serum; Rm, *Reithrodontomys megalotis* serum; Anti-SNV, serum from patients with HPS or *P. maniculatus* infected with Sin Nombre virus; Anti-CARV, serum from Carrizal virus-infected *R. sumichrasti*; Anti-HUIV, serum from Huitzilac virus-infected *R. megalotis*. The OD value was corrected by subtracting the OD value with negative control antigen. The ELISA was performed three times in duplicate and the bar shows the mean values of a representative experiment.

4. Discussion

New World hantaviruses were divided into five groups and groups 2, 3 and 4 were further divided into five, three and three subgroups, respectively, by comparison of amino acid sequence identity (aa 230–302) of the variable region of NP (Table 1). These hantaviruses were also grouped into five corresponding groups geographically and phylogenetically (Fig. 1A and B). This classification corresponds basically to a previous study in which hantaviruses were classified by comparison of the entire amino acid sequences of S and M segments (Maes et al., 2009).

As shown in Fig. 1B, groups 1, 2 and 3 include hantaviruses that are pathogenic to humans. On the other hand, hantaviruses in groups 4 and 5 have not been reported to cause disease in humans. Thus, serotyping among infections is important for clinical diagnosis and epidemiological surveillance in regions where two or more hantaviruses co-circulate.

In this study, the applicability of N-terminally truncated rNPs of those three hantavirus species as antigens for serotyping ELISA to differentiate SNV and CARV infection was investigated. Sera from SNV- and CARV-infected rodents or humans reacted strongly to homologous antigens. In contrast, these sera reacted weakly to heterologous antigens including BCCV trNP100 (Fig. 3). The trNP100s of SNV, CARV and BCCV could distinguish human and rodent sera. Together with the work reported previously (Koma et al., 2010), trNP100s of SNV (group 1), ANDV (group 2), BCCV (group 3) and CARV (group 4) have been prepared. They can distinguish infections between groups except for group 5 in the New World hantaviruses. On the other hand, the whole rNPs of SNV, BCCV and CARV expressed by *E. coli* reacted strongly at almost equal levels to all of the heterologous sera. Therefore, screening ELISA using whole rNPs from any one of the New World hantaviruses followed by serotyping ELISA using the trNPs is recommended as a rapid and practical system for hantavirus seroepidemiology.

There have been few reports on MAbs to NP of New World hantaviruses (Tischler et al., 2008). Consequently, antigenic characterization of rNPs was indirectly confirmed using MAbs to Old World hantaviruses by IFA. MAbs that recognize immunodominant epitopes of the N-terminus of NP reacted to whole rNPs and trNP50s but not to trNP100s in IFA (Table 2). These results support those of previous studies indicating that the first 100 aa of the N-terminus of NP possess immunodominant, cross-reactive epitopes (Elgh et al.,

1996; Gott et al., 1997; Vapalahti et al., 1995; Yamada et al., 1995) and suggest that these cross-reactive epitopes are conserved highly among both the New World and the Old World hantaviruses. Only MAb E5/G6 that bound to the conserved linear epitope recognized commonly all rNPs (Okumura et al., 2004). Thus, MAb E5/G6 could be used as a capture antibody for both the rNPs of New World and Old World hantaviruses and also as a detection antibody.

It has been reported that conformation-dependent, serotype-specific epitopes in NP are located from aa 205 to 290 (Yoshimatsu et al., 1996, 2003). The major linear epitopes in NP have been reported to be located at the N-terminus (Elgh et al., 1996; Gott et al., 1997; Yamada et al., 1995). Therefore, truncated rNPs that lacked only a minimal region were applied and trNP50s were prepared as well as trNP50s for Old World hantaviruses that were reported previously (Araki et al., 2001). However, trNP50s of SNV and CARV were found as monomeric NP in the competitive-sandwich ELISA (Fig. 2). Furthermore, the trNP50s showed higher cross-reactivity with each other (data not shown). Therefore, trNP50s of those were not applicable for serotyping antigens. The results indicated that trNP50s still possessed cross-reactivity to heterologous sera. In contrast, trNP100s of SNV, CARV and BCCV were detected as multimers (Fig. 2). The whole rNP and trNP50 of BCCV reacted to detector MAb E5/G6 in the competitive-sandwich ELISA, but the reaction was weak in comparison to reaction of the BCCV trNP100. These results support results of other studies indicating that the first 100 aa of the N-terminus did not contribute to NP-NP interaction (Kaukinen et al., 2004; Yoshimatsu et al., 2003).

In terms of public health, it is important to develop rapid, safe and convenient serotyping methods for epidemiological surveillance and studies. This system will become a valuable tool for surveying human and rodent cases of New World hantavirus infections.

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