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ORIGINAL ARTICLE

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

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

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Introduction

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

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

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

Materials and methods

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

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

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

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

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

Preparation of TPMV-N-protein specific monoclonal antibodies

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

Indirect immunofluorescence assay (IFA)

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

Table 1 Characteristics of monoclonal antibodies produced in this study

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

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

* The subclass and light chain of B5H9 were not defined

Enzyme-linked immunosorbent assay (ELISA)

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

Western blot test

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

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

Purification and biotinylation of the immunoglobulin fraction

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

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

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

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

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

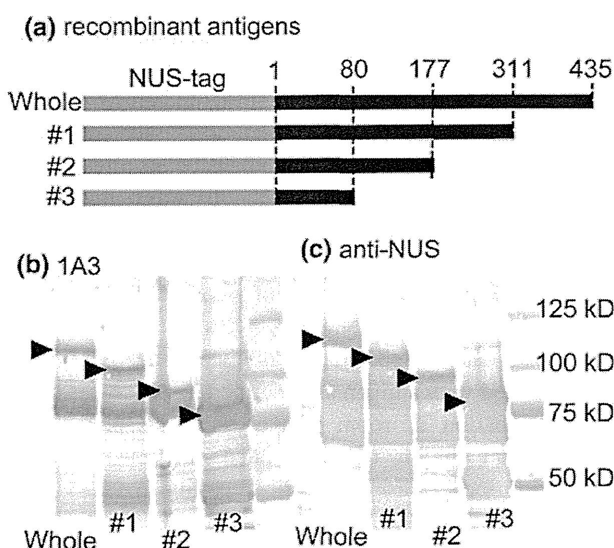

Fig. 1 Schematic representation of the structure (**a**) and western blot reactivity of full-length and truncated Thottapalayam virus nucleocapsid protein derivatives with TPMV-specific monoclonal antibody (mAb) 1A3 (**b**) and anti-NUS mAb (**c**)

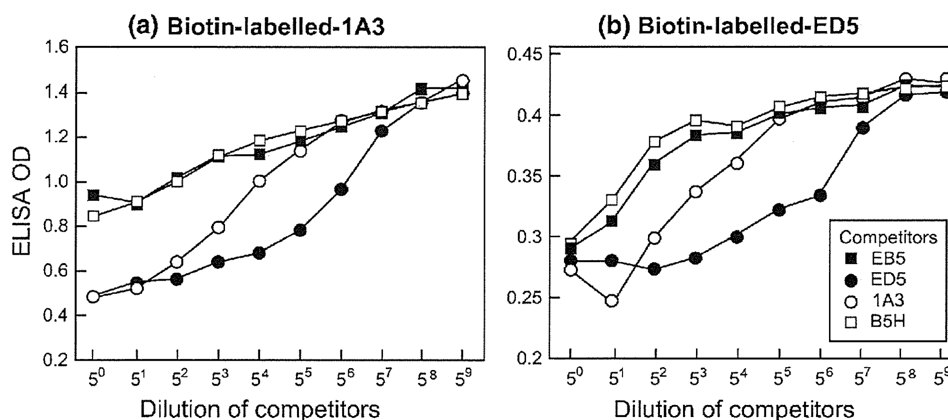

Fig. 2 Competitive binding assay with biotin-labeled mAbs 1A3 (**a**) and ED5 (**b**). Absorbance values (OD) at 405 nm are shown on the y-axis and are compared with those in the absence of competitive antibody. Dilutions of the competitive antibodies are shown on the x-axis. The relative binding avidity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

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

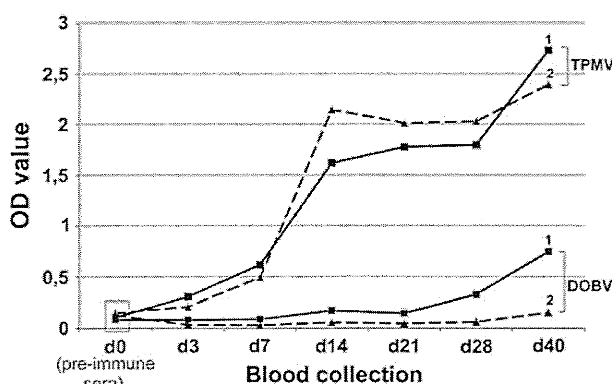

Fig. 3 ELISA reactivity of the sera from two experimentally TPMV-infected Asian house shrews against yeast-expressed TPMV and DOBV N proteins. Absorbance values (OD) of the ELISAs measured at 405 nm are shown on the y-axis. Time points after the experimental infection are given on the x-axis. Reactivities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

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

Discussion

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

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

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

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

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

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

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References

1. Arai S, Song JW, Sumibcay L, Bennett SN, Nerurkar VR, Parmenter C, Cook JA, Yates TL, Yanagihara R (2007) Hantavirus in northern short-tailed shrew, United States. Emerg Infect Dis 13:1420–1423
2. Arai S, Bennett SN, Sumibcay L, Cook JA, Song JW, Hope A, Parmenter C, Nerurkar VR, Yates TL, Yanagihara R (2008) Phylogenetically distinct hantaviruses in the masked shrew (Sorex cinereus) and dusky shrew (Sorex monticolus) in the United States. Am J Trop Med Hyg 78:348–351
3. Arikawa J, Schmaljohn AL, Dalrymple JM, Schmaljohn CS (1989) Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. J Gen Virol 70(Pt 3):615–624
4. Biltueva LS, Rogatcheva MB, Perelman PL, Borodin PM, Oda SI, Koyasu K, Harada M, Zima J, Graphodatsky AS (2001) Chromosomal phylogeny of certain shrews of the genera Crocidura and Suncus (Insectivora). J Zoological Syst Evol Res 39:69–76
5. Carey DE, Reuben R, Panicker KN, Shope RE, Myers RM (1971) Thottapalayam virus: a presumptive arbovirus isolated from a shrew in India. Indian J Med Res 59:1758–1760
6. Chu YK, Rossi C, Leduc JW, Lee HW, Schmaljohn CS, Dalrymple JM (1994) Serological relationships among viruses in the Hantavirus genus, family Bunyaviridae. Virology 198:196–204

7. Dantas JR Jr, Okuno Y, Asada H, Tamura M, Takahashi M, Tanishita O, Takahashi Y, Kurata T, Yamanishi K (1986) Characterization of glycoproteins of viruses causing hemorrhagic fever with renal syndrome (HFRS) using monoclonal antibodies. Virology 151:379–384
8. Dzagurova T, Tkachenko E, Slonova R, Ivanov L, Ivanidze E, Markeshin S, Dekonenko A, Niklasson B, Lundkvist A (1995) Antigenic relationships of hantavirus strains analysed by monoclonal antibodies. Arch Virol 140:1763–1773
9. Elgh F, Wadell G, Juto P (1995) Comparison of the kinetics of Puumala virus specific IgM and IgG antibody responses in nephropathia epidemica as measured by a recombinant antigen-based enzyme-linked immunosorbent assay and an immunofluorescence test. J Med Virol 45:146–150
10. Franko MC, Gibbs CJ Jr, Lee PW, Gajdusek DC (1983) Monoclonal antibodies specific for Hantaan virus. P Natl Acad Sci USA 80:4149–4153
11. Jonsson CB, Schmaljohn CS (2001) Replication of hantaviruses. Curr Top Microbiol Immunol 256:15–32
12. Kang HJ, Arai S, Hope AG, Song JW, Cook JA, Yanagihara R (2009) Genetic diversity and phylogeography of Seewis virus in the Eurasian common shrew in Finland and Hungary. Virol J 6:208
13. Kang HJ, Arai S, Hope AG, Cook JA, Yanagihara R (2010) Novel hantavirus in the flat-skulled shrew (Sorex roboratus). Vector Borne Zoonotic Dis 10:593–597
14. Kang HJ, Bennett SN, Hope AG, Cook JA, Yanagihara R (2011) Shared ancestry between a newfound mole-borne hantavirus and hantaviruses harbored by cricetid rodents. J Virol 85(15):7496–7503
15. Klempa B, Fichet-Calvet E, Lecompte E, Auste B, Aniskin V, Meisel H, Denys C, Koivogui L, ter Meulen J, Kruger DH (2006) Hantavirus in African wood mouse, Guinea. Emerg Infect Dis 12:838–840
16. Klempa B, Fichet-Calvet E, Lecompte E, Auste B, Aniskin V, Meisel H, Barriere P, Koivogui L, ter Meulen J, Kruger DH (2007) Novel hantavirus sequences in Shrew, Guinea. Emerg Infect Dis 13:520–522
17. Koch J, Liang MF, Queitsch I, Kraus AA, Bautz EKF (2003) Human recombinant neutralizing antibodies against Hantaan virus G2 protein. Virology 308:64–73
18. Kucinskaite-Kodze I, Petraityte-Burneikiene R, Zvirbliene A, Hjelle B, Medina RA, Gedvilaite A, Razanskiene A, Schmidt-Chanasit J, Mertens M, Padula P, Sasnauskas K, Ulrich RG (2011) Characterization of monoclonal antibodies against hantavirus nucleocapsid protein and their use for immunohistochemistry on rodent and human samples. Arch Virol 156:443–456
19. Liang M, Guttieri M, Lundkvist A, Schmaljohn C (1997) Baculovirus expression of a human G2-specific, neutralizing IgG monoclonal antibody to Puumala virus. Virology 235:252–260
20. Liang M, Mahler M, Koch J, Ji Y, Li D, Schmaljohn C, Bautz EK (2003) Generation of an HFRS patient-derived neutralizing recombinant antibody to Hantaan virus G1 protein and definition of the neutralizing domain. J Med Virol 69:99–107
21. Lundkvist A, Fatouros A, Niklasson B (1991) Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies. J Gen Virol 72(Pt 9):2097–2103
22. Lundkvist A, Niklasson B (1992) Bank vole monoclonal antibodies against Puumala virus envelope glycoproteins: identification of epitopes involved in neutralization. Arch Virol 126:93–105
23. Lundkvist A, Horling J, Athlin L, Rosen A, Niklasson B (1993) Neutralizing human monoclonal antibodies against Puumala virus, causative agent of nephropathia epidemica: a novel method using antigen-coated magnetic beads for specific B cell isolation. J Gen Virol 74(Pt 7):1303–1310
24. Lundkvist A, Vapalahti O, Plyusnin A, Sjolander KB, Niklasson B, Vaheri A (1996) Characterization of Tula virus antigenic determinants defined by monoclonal antibodies raised against baculovirus-expressed nucleocapsid protein. Virus Res 45:29–44
25. Lundkvist A, Meisel H, Koletzki D, Lankinen H, Cifire F, Geldmacher A, Sibold C, Gott P, Vaheri A, Kruger DH, Ulrich R (2002) Mapping of B-cell epitopes in the nucleocapsid protein of Puumala hantavirus. Viral Immunol 15:177–192
26. Mazzarotto GA, Raboni SM, Stella V, Carstensen S, de Noronha L, Levis S, Zanluca C, Zanetti CR, Bordignon J, Duarte dos Santos CN (2009) Production and characterization of monoclonal antibodies against the recombinant nucleoprotein of Araucaria hantavirus. J Virol Methods 162:96–100
27. Mertens M, Hofmann J, Petraityte-Burneikiene R, Ziller M, Sasnauskas K, Friedrich R, Niederstrasser O, Kruger DH, Groschup MH, Petri E, Werdermann S, Ulrich RG (2011) Seroprevalence study in forestry workers of a non-endemic region in eastern Germany reveals infections by Tula and Dobrava-Belgrade hantaviruses. Med Microbiol Immunol 200:263–268
28. Mertens M, Kindler E, Emmerich P, Esser J, Wagner-Wiening C, Wolfel R, Petraityte-Burneikiene R, Schmidt-Chanasit J, Zvirbliene A, Groschup MH, Dobler G, Pfeffer M, Heckel G, Ulrich RG, Essbauer SS (2011) Phylogenetic analysis of Puumala virus subtype Bavaria, characterization and diagnostic use of its recombinant nucleocapsid protein. Virus Genes
29. Motokawa M, Suzuki H, Harada M, Lin L-K, Koyasu K, S-I Oda (2000) Phylogenetic relationships among East Asian species of Crocidura (Mammalia, Insectivora) inferred from mitochondrial cytochrome b gene sequences. Zoological Sci (Tokyo) 17: 497–504
30. Okumura M, Yoshimatsu K, Kumperasart S, Nakamura I, Ogino M, Taruishi M, Sungdee A, Pattamadilok S, Ibrahim IN, Erlina S, Agui T, Yanagihara R, Arikawa J (2007) Development of serological assays for Thottapalayam virus, an insectivore-borne Hantavirus. Clin Vaccine Immunol 14:173–181
31. Plyusnin A, Morzunov SP (2001) Virus evolution and genetic diversity of hantaviruses and their rodent hosts. Curr Top Microbiol Immunol 256:47–75
32. Ramsden C, Holmes EC, Charleston MA (2009) Hantavirus evolution in relation to its rodent and insectivore hosts: no evidence for codivergence. Mol Biol Evol 26:143–153
33. Razanskiene A, Schmidt J, Geldmacher A, Ritzi A, Niedrig M, Lundkvist A, Kruger DH, Meisel H, Sasnauskas K, Ulrich R (2004) High yields of stable and highly pure nucleocapsid proteins of different hantaviruses can be generated in the yeast Saccharomyces cerevisiae. J Biotechnol 111:319–333
34. Ruo SL, Sanchez A, Elliott LH, Brammer LS, McCormick JB, Fisher-Hoch SP (1991) Monoclonal antibodies to three strains of hantaviruses: Hantaan, R22, and Puumala. Arch Virol 119:1–11
35. Salonen EM, Parren PW, Graus YF, Lundkvist A, Fisicaro P, Vapalahti O, Kallio-Kokko H, Vaheri A, Burton DR (1998) Human recombinant Puumala virus antibodies: cross-reaction with other hantaviruses and use in diagnostics. J Gen Virol 79(Pt 4):659–665
36. Schmidt-Chanasit J, Essbauer S, Petraityte R, Yoshimatsu K, Tackmann K, Conraths FJ, Sasnauskas K, Arikawa J, Thomas A, Pfeffer M, Scharninghausen JJ, Splettstoesser W, Wenk M, Heckel G, Ulrich RG (2010) Extensive host sharing of central European Tula virus. J Virol 84:459–474
37. Schlegel M, Radosa L, Rosenfeld UM, Schmidt S, Triebenbacher C, Löhr PW, Fuchs D, Heroldová M, Jánová E, Stanko M, Mošanský L, Fričová J, Pejčoch M, Suchomel J, Purchart L, Groschup MH, Krüger DH, Klempa B, Ulrich RG (2012) Broad geographical distribution and high genetic diversity of shrew-borne Seewis hantavirus in Central Europe. Virus Genes [Epub ahead of print]

38. Song JW, Baek LJ, Schmaljohn CS, Yanagihara R (2007) Thottapalayam virus, a prototype shrewborne hantavirus. Emerg Infect Dis 13:980–985
39. Song JW, Gu SH, Bennett SN, Arai S, Puorger M, Hilbe M, Yanagihara R (2007) Seewis virus, a genetically distinct hantavirus in the Eurasian common shrew (Sorex araneus). Virol J 4:114
40. Song JW, Kang HJ, Gu SH, Moon SS, Bennett SN, Song KJ, Baek LJ, Kim HC, O'Guinn ML, Chong ST, Klein TA, Yanagihara R (2009) Characterization of Imjin virus, a newly isolated hantavirus from the Ussuri white-toothed shrew (Crocidura lasiura). J Virol 83:6184–6191
41. Sugiyama K, Morikawa S, Matsuura Y, Tkachenko EA, Morita C, Komatsu T, Akao Y, Kitamura T (1987) Four serotypes of haemorrhagic fever with renal syndrome viruses identified by polyclonal and monoclonal antibodies. J Gen Virol 68(Pt 4): 979–987
42. Tischler ND, Rosemblatt M, Valenzuela PD (2008) Characterization of cross-reactive and serotype-specific epitopes on the nucleocapsid proteins of hantaviruses. Virus Res 135:1–9
43. Yadav PD, Vincent MJ, Nichol ST (2007) Thottapalayam virus is genetically distant to the rodent-borne hantaviruses, consistent with its isolation from the Asian house shrew (Suncus murinus). Virol J 4:80
44. Yamada T, Hjelle B, Lanzi R, Morris C, Anderson B, Jenison S (1995) Antibody responses to Four Corners hantavirus infections in the deer mouse (Peromyscus maniculatus): identification of an immunodominant region of the viral nucleocapsid protein. J Virol 69:1939–1943
45. Yamanishi K, Dantas JR Jr, Takahashi M, Yamanouchi T, Domae K, Takahashi Y, Tanishita O (1984) Antigenic differences between two viruses, isolated in Japan and Korea, that cause hemorrhagic fever with renal syndrome. J Virol 52:231–237
46. Yashina LN, Abramov SA, Gutorov VV, Dupal TA, Krivopalov AV, Panov VV, Danchinova GA, Vinogradov VV, Luchnikova EM, Hay J, Kang HJ, Yanagihara R (2010) Seewis virus: phylogeography of a shrew-borne hantavirus in Siberia, Russia. Vector Borne Zoonotic Dis 10:585–591
47. Yoshimatsu K, Arikawa J, Kariwa H (1993) Application of a recombinant baculovirus expressing hantavirus nucleocapsid protein as a diagnostic antigen in IFA test: cross reactivities among 3 serotypes of hantavirus which causes hemorrhagic fever with renal syndrome (HFRS). J Vet Med Sci 55:1047–1050
48. Yoshimatsu K, Arikawa J, Yoshida R, Li H, Yoo YC, Kariwa H, Hashimoto N, Kakinuma M, Nobunaga T, Azuma I (1995) Production of recombinant hantavirus nucleocapsid protein expressed in silkworm larvae and its use as a diagnostic antigen in detecting antibodies in serum from infected rats. Lab Anim Sci 45:641–646
49. Yoshimatsu K, Arikawa J, Tamura M, Yoshida R, Lundkvist A, Niklasson B, Kariwa H, Azuma I (1996) Characterization of the nucleocapsid protein of Hantaan virus strain 76–118 using monoclonal antibodies. J Gen Virol 77(Pt 4):695–704
50. Yu S, Liang M, Fan B, Xu H, Li C, Zhang Q, Li D, Tang B, Li S, Dai Y, Wang M, Zheng M, Yan B, Zhu Q, Li N (2006) Maternally derived recombinant human anti-hantavirus monoclonal antibodies are transferred to mouse offspring during lactation and neutralize virus in vitro. J Virol 80:4183–4186
51. Zoller LG, Yang S, Gott P, Bautz EK, Darai G (1993) A novel mu-capture enzyme-linked immunosorbent assay based on recombinant proteins for sensitive and specific diagnosis of hemorrhagic fever with renal syndrome. J Clin Microbiol 31:1194–1199
52. Zvirbliene A, Samonskyte L, Gedvilaite A, Voronkova T, Ulrich R, Sasnauskas K (2006) Generation of monoclonal antibodies of desired specificity using chimeric polyomavirus-derived virus-like particles. J Immunol Methods 311:57–70

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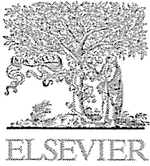

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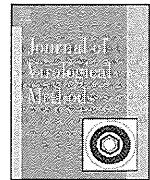

Development of a serotyping enzyme-linked immunosorbent assay system based on recombinant truncated hantavirus nucleocapsid proteins for New World hantavirus infection

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ABSTRACT

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.
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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

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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 Genetyx-Mac 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

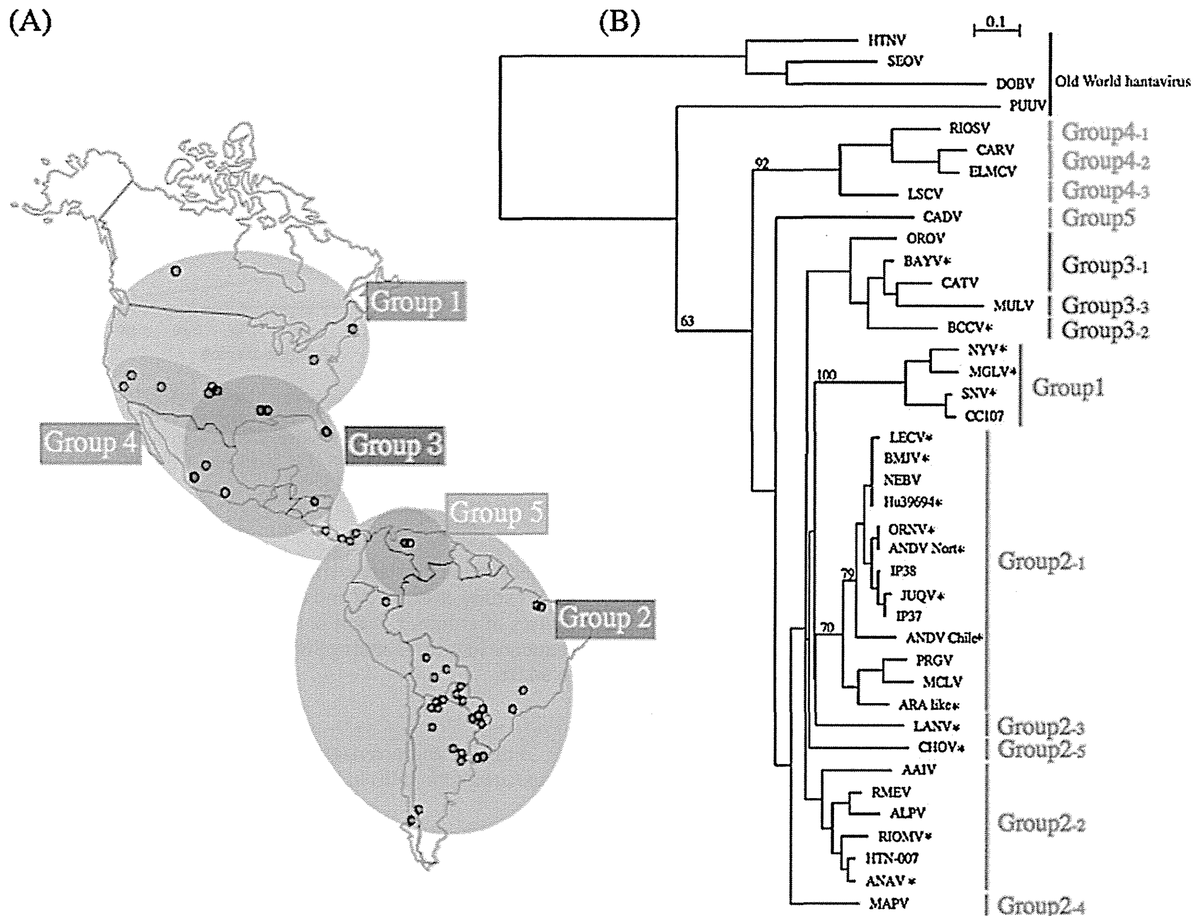

Fig. 1. Distribution map of grouped New World hantaviruses and phylogenetic tree for New World hantavirus. (A) The map represents the geographical distribution of grouped New World hantaviruses. (B) Phylogenetic tree for New World hantavirus. Neighbor-joining phylogenetic analysis was performed on the 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 HisTrap 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 GB04 and EC01 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%.

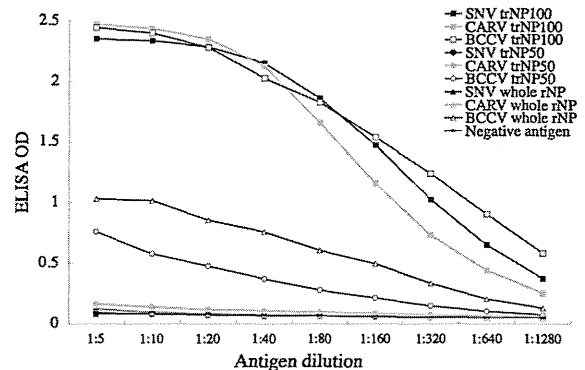

Fig. 2. Multimerization of rNPs in competitive-sandwich ELISA. Antigens were captured and detected with MAb E5/G6. Each antigen was diluted from 1:5 to 1:1280 and subjected to capture ELISA. Positive reaction with this ELISA indicates that the antigens are forming a multimer. The ELISA was performed 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, GB04, C16D11, EC02 and EC01) 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 EC02. The trNP50s of SNV, CARV and BCCV lacked reactivity with 5 of the N-terminal specific MAbs (2E12, 4C3, 4E5, EC02 and EC01) but still reacted with MAb GB04, 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.

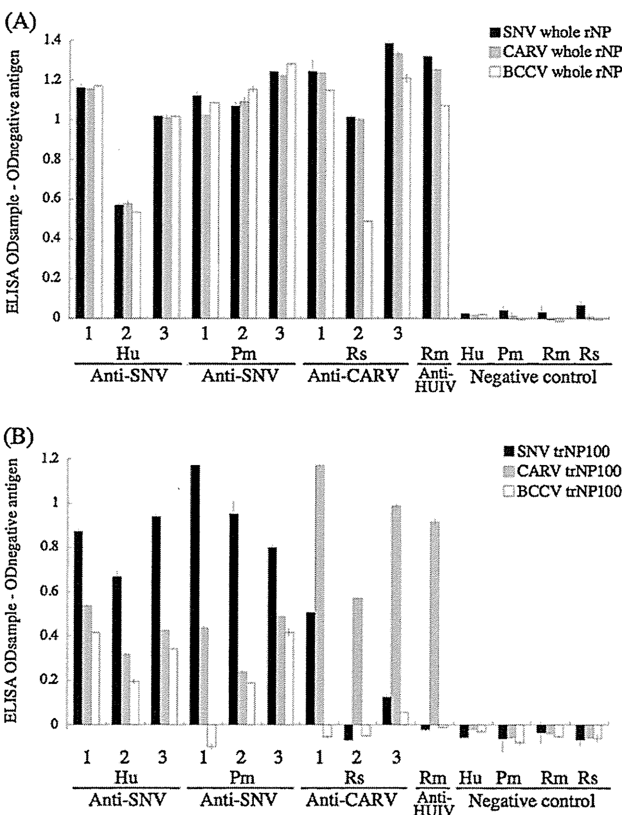

Fig. 3. Reaction patterns of hantavirus-positive and -negative sera in ELISA. (A) Reaction patterns of whole rNPs (SNV, CARV and BCCV) with human and rodent sera in ELISA. (B) Reaction patterns of trNP100s (SNV, CARV and BCCV) with human and rodent sera in ELISA. Abbreviations: Hu, human serum; Pm, *Peromyscus 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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References

Araki, K., Yoshimatsu, K., Ogino, M., Ebihara, H., Lundkvist, A., Kariwa, H., Takashima, I., Arikawa, J., 2001. Truncated hantavirus nucleocapsid proteins for serotyping Hantaan, Seoul, and Dobrava hantavirus infections. Journal of Clinical Microbiology 39, 2397–2404.

Botten, J., Mirowsky, K., Kusewitt, D., Bharadwaj, M., Yee, J., Ricci, R., Feddersen, R.M., Hjelle, B., 2000. Experimental infection model for Sin Nombre hantavirus in the deer mouse (*Peromyscus maniculatus*). Proceedings of the National Academy of Sciences of the United States of America 97, 10578–10583.

Elgh, F., Lundkvist, A., Alexeyev, O.A., Wadell, G., Juto, P., 1996. A major antigenic domain for the human humoral response to Puumala virus nucleocapsid protein is located at the amino-terminus. Journal of Virological Methods 59, 161–172.

Elliott, R.M., 1990. Molecular biology of the Bunyaviridae. Journal of General Virology 71, 501–522.

Elliott, R.M., Bouloy, M., Calisher, C.H., Goldbach, R., Moyer, J.T., Nichol, S.T., Petterson, R., Plyusin, A., Schmaljohn, C., 2000. Bunyaviridae. In: van Regenmortel, C.M.F.M.H.V., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Virus Taxonomy: The Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp. 599–621.

Gott, P., Zoller, L., Darai, G., Bautz, E.K., 1997. A major antigenic domain of hantaviruses is located on the aminoproximal site of the viral nucleocapsid protein. Virus Genes 14, 31–40.

Hjelle, B., Jenison, S., Torrez-Martinez, N., Yamada, T., Nolte, K., Zumwalt, R., MacInnes, K., Myers, G., 1994. A novel hantavirus associated with an outbreak of fatal respiratory disease in the southwestern United States: evolutionary relationships to known hantaviruses. Journal of Virology 68, 592–596.

Hughes, A.L., Friedman, R., 2000. Evolutionary diversification of protein-coding genes of hantaviruses. Molecular Biology and Evolution 17, 1558–1568.

Jonsson, C.B., Figueiredo, L.T., Vapalahti, O., 2010. A global perspective on hantavirus ecology, epidemiology, and disease. Clinical Microbiology Reviews 23, 412–441.

Kariwa, H., Yoshida, H., Sanchez-Hernandez, C., Romero-Almaraz Mde, L., Almazan-Catalan, J.A., Ramos, C., Miyashita, D., Seto, T., Takano, A., Totani, M., Murata, R., Saasa, N., Ishizuka, M., Sanada, T., Yoshii, K., Yoshimatsu, K., Arikawa, J., Takashima, I., 2012. Genetic diversity of hantaviruses in Mexico: identification of three novel hantaviruses from Neotominae rodents. Virus Research 163, 486–494.

Kariwa, H., Yoshimatsu, K., Arikawa, J., 2007. Hantavirus infection in East Asia. Comparative Immunology, Microbiology and Infectious Diseases 30, 341–356.

Kaukinen, P., Koistinen, V., Vapalahti, O., Vaheri, A., Plyusnin, A., 2001. Interaction between molecules of hantavirus nucleocapsid protein. Journal of General Virology 82, 1845–1853.

Kaukinen, P., Kumar, V., Tulimaki, K., Engelhardt, P., Vaheri, A., Plyusnin, A., 2004. Oligomerization of Hantavirus N protein: C-terminal alpha-helices interact to form a shared hydrophobic space. Journal of Virology 78, 13669–13677.

Koma, T., Yoshimatsu, K., Pini, N., Safronetz, D., Taruishi, M., Levis, S., Endo, R., Shimizu, K., Yasuda, S.P., Ebihara, H., Feldmann, H., Enria, D., Arikawa, J., 2010. Truncated hantavirus nucleocapsid proteins for serotyping Sin Nombre, Andes, and Laguna Negra hantavirus infections in humans and rodents. Journal of Clinical Microbiology 48, 1635–1642.

Lee, H.W., van der Groen, G., 1989. Hemorrhagic fever with renal syndrome. Progress in Medical Virology 36, 62–102.

Lundkvist, A., Fatouros, A., Niklasson, B., 1991. Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies. Journal of General Virology 72, 2097–2103.

Maes, P., Klempa, B., Clement, J., Matthijnssens, J., Gajdusek, D.C., Kruger, D.H., Van Ranst, M., 2009. A proposal for new criteria for the classification of hantaviruses, based on S and M segment protein sequences. Infection, Genetics and Evolution 9, 813–820.

Meyer, B.J., Schmaljohn, C.S., 2000. Persistent hantavirus infections: characteristics and mechanisms. Trends in Microbiology 8, 61–67.

Morii, M., Yoshimatsu, K., Arikawa, J., Zhou, G., Kariwa, H., Takashima, I., 1998. Antigenic characterization of Hantaan and Seoul virus nucleocapsid proteins expressed by recombinant baculovirus: application of a truncated protein, lacking an antigenic region common to the two viruses, as a serotyping antigen. Journal of Clinical Microbiology 36, 2514–2521.

Nakamura, I., Yoshimatsu, K., Lee, B.H., Okumura, M., Taruishi, M., Araki, K., Kariwa, H., Takashima, I., Arikawa, J., 2008. Development of a serotyping ELISA system for Thailand virus infection. Archives of Virology 153, 1537–1542.

Okumura, M., Yoshimatsu, K., Araki, K., Lee, B.H., Asano, A., Agui, T., Arikawa, J., 2004. Epitope analysis of monoclonal antibody E5/G6, which binds to a linear epitope in the nucleocapsid protein of hantaviruses. Archives of Virology 149, 2427–2434.

Perriere, G., Gouy, M., 1996. WWW-query: an on-line retrieval system for biological sequence banks. Biochimie 78, 364–369.

Peters, C.J., Khan, A.S., 2002. Hantavirus pulmonary syndrome: the new American hemorrhagic fever. Clinical Infectious Diseases 34, 1224–1231.

Ravkov, E.V., Rollin, P.E., Ksiazek, T.G., Peters, C.J., Nichol, S.T., 1995. Genetic and serologic analysis of Black Creek Canal virus and its association with human disease and Sigmodon hispidus infection. Virology 210, 482–489.

Ruo, S.L., Sanchez, A., Elliott, L.H., Brammer, L.S., McCormick, J.B., Fisher, H.-S., 1991. Monoclonal antibodies to three strains of hantaviruses: Hantaan, R22, and Puumala. Archives of Virology 119, 1–11.

Schmaljohn, C., Hjelle, B., 1997. Hantaviruses: a global disease problem. Emerging Infectious Diseases 3, 95–104.

Schmaljohn, C.S., 1996. Molecular biology of hantaviruses. In: Elliott, R.M. (Ed.), The Bunyaviridae. Plenum Press, New York, pp. 63–90.

Tischler, N.D., Rosemblatt, M., Valenzuela, P.D., 2008. Characterization of cross-reactive and serotype-specific epitopes on the nucleocapsid proteins of hantaviruses. Virus Research 135, 1–9.

Vapalahti, O., Kallio-Kokko, H., Narvanen, A., Julkunen, I., Lundkvist, A., Plyusnin, A., Lehvaslaiho, H., Brummer-Korvenkontio, M., Vaheri, A., Lankinen, H., 1995. Human B-cell epitopes of Puumala virus nucleocapsid protein, the major antigen in early serological response. Journal of Medical Virology 46, 293–303.

Yamada, T., Hjelle, B., Lanzi, R., Morris, C., Anderson, B., Jenison, S., 1995. Antibody responses to Four Corners hantavirus infections in the deer mouse (*Peromyscus maniculatus*): identification of an immunodominant region of the viral nucleocapsid protein. Journal of Virology 69, 1939–1943.

Yasuda, S.P., Yoshimatsu, K., Koma, T., Shimizu, K., Endo, R., Isozumi, R., Arikawa, J., 2012. Application of truncated nucleocapsid protein (N) for serotyping ELISA of murinae-associated hantavirus infection in rats. Journal of Veterinary Medical Science 74, 215–219.

Yoshimatsu, K., Arikawa, J., Kariwa, H., 1993. Application of a recombinant baculovirus expressing hantavirus nucleocapsid protein as a diagnostic antigen in IFA test: cross reactivities among 3 serotypes of hantavirus which causes

hemorrhagic fever with renal syndrome (HFRS). Journal of Veterinary Medical Science 55, 1047–1050.
Yoshimatsu, K., Arikawa, J., Tamura, M., Yoshida, R., Lundkvist, A., Niklasson, B., Kariwa, H., Azuma, I., 1996. Characterization of the nucleocapsid protein of Hantaan virus strain 76-118 using monoclonal antibodies. Journal of General Virology 77, 695–704.
Yoshimatsu, K., Arikawa, J., Yoshida, R., Li, H., Yoo, Y.C., Kariwa, H., Hashimoto, N., Kakinuma, M., Nobunaga, T., Azuma, I., 1995. Production of recombinant hantavirus nucleocapsid protein expressed in silkworm larvae and its use as a diagnostic antigen in detecting antibodies in serum from infected rats. Laboratory Animal Science 45, 641–646.
Yoshimatsu, K., Lee, B.H., Araki, K., Morimatsu, M., Ogino, M., Ebihara, H., Arikawa, J., 2003. The multimerization of hantavirus nucleocapsid protein depends on type-specific epitopes. Journal of Virology 77, 943–952.
Zeier, M., Handermann, M., Bahr, U., Rensch, B., Muller, S., Kehm, R., Muranyi, W., Darai, G., 2005. New ecological aspects of hantavirus infection: a change of a paradigm and a challenge of prevention – a review. Virus Genes 30, 157–180.

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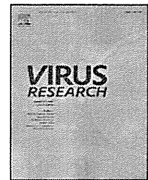

Serial passage of a street rabies virus in mouse neuroblastoma cells resulted in attenuation: Potential role of the additional *N*-glycosylation of a viral glycoprotein in the reduced pathogenicity of street rabies virus☆

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ABSTRACT

Street rabies viruses are field isolates known to be highly neurotropic. However, the viral elements related to their pathogenicity have yet to be identified at the nucleotide or amino acid level. Here, through 30 passages in mouse neuroblastoma NA cells, we have established an attenuated variant of street rabies virus strain 1088, originating from a rabid woodchuck followed by 2 passages in the brains of suckling mice. The variant, 1088-N30, was well adapted to NA cells and highly attenuated in adult mice after intramuscular (i.m.) but not intracerebral (i.c.) inoculations. 1088-N30 had seven nucleotide substitutions, and the R196S mutation of the G protein led to an additional *N*-glycosylation. Street viruses usually possess one or two *N*-glycosylation sites on the G protein, 1088 has two, while an additional *N*-glycosylation site is observed in laboratory-adapted strains. We also established a cloned variant 1088-N4#14 by limiting dilution. Apart from the R196S mutation, 1088-N4#14 possessed only one amino acid substitution in the P protein, which is found in several field isolates. 1088-N4#14 also efficiently replicated in NA cells and was attenuated in adult mice after i.m. inoculations, although it was more pathogenic than 1088-N30. The spread of 1088-N30 in the brain was highly restricted after i.m. inoculations, although the pattern of 1088-N4#14's spread was intermediate between that of the parental 1088 and 1088-N30. Meanwhile, both variants strongly induced humoral immune responses in mice compared to 1088. Our results indicate that the additional *N*-glycosylation is likely related to the reduced pathogenicity. Taken together, we propose that the number of *N*-glycosylation sites in the G protein is one of the determinants of the pathogenicity of street rabies viruses.

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1. Introduction

Rabies virus is a causative agent of rabies, a fatal encephalitis in mammals, including humans. Rabies viruses belong to the genus *Lyssavirus*, family *Rhabdoviridae*. They are bullet-shaped enveloped viruses and have a nonsegmented negative-strand RNA genome about 12 kb in length. The genome encodes five viral proteins: the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large protein (L). The G protein is the only viral protein that is glycosylated and exposed at the surface of the virion (Wunner, 2007). It is also responsible for the interaction with receptors to enter into target cells and the induction of a humoral immune response, such as the production of virus-neutralizing antibodies (Wunner, 2007). Moreover, studies show that the G protein is the major factor responsible for the

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; CVS, challenge virus standard; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FCS, fetal calf serum; FFU, focus-forming units; i.c., intracerebral; i.m., intramuscular; IU, international units; LD_{50}, 50% lethal dose; MAb, monoclonal antibody; MOI, multiplicity of infection; p.i., post-inoculation; SD, standard deviation; SHBRV, silver-haired bat rabies virus; USA, United States of America; VNA, virus neutralizing antibodies; WNV, West Nile virus.

☆ The sequences determined in the present study have been deposited in the GenBank/EMBL/DDBJ databases (GenBank ID: AB645847).

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pathogenesis of rabies viruses (Dietzschold et al., 1983, 1985; Etessami et al., 2000; Ito et al., 2001b; Morimoto et al., 1999, 2000; Seif et al., 1985; Takayama-Ito et al., 2004, 2006a,b; Tuffereau et al., 1989).

Rabies viruses are generally classified into two categories, street viruses (field isolates) and fixed viruses (laboratory-adapted strains). Street viruses are known to be more pathogenic than fixed viruses after peripheral infections. The first fixed virus was established by Pasteur through the repeated passaging of a street virus in rabbits via intracerebral (i.c.) inoculations (Baer, 2007; Lepine, 1938). Although fixed viruses are generally neurovirulent (pathogenic to animals after an i.c. inoculation), they have different characteristics from street viruses, such as regularity and shortening of the incubation period, stabilization of virulence, and a reduction or loss of infectivity after peripheral inoculations (Lepine, 1938).

Some groups have investigated the pathogenecity of street viruses using the silver-haired bat-associated rabies virus (SHBRV), a highly pathogenic and neuroinvasive street virus (Dietzschold et al., 2000). Compared to attenuated fixed strains, SHBRV is a poor inducer of innate immune responses in mice after an intramuscular (i.m.) inoculation (Wang et al., 2005), and mice infected with SHBRV intradermally failed to open the blood–brain barrier (BBB) and to recruit immune effectors into the CNS (Roy and Hooper, 2007; Roy et al., 2007). The maintenance of the BBB permeability was also observed for several terrestrial animal-derived viruses (Kuang et al., 2009; Roy and Hooper, 2008). In addition, the study of chimeric viruses generated from SHBRV and the fixed virus SAD B19 has been identified the M and G proteins as related to the pathogenecity of SHBRV; the M and G proteins enhanced viral internalization and spread in cultured cells (Faber et al., 2004; Pulmanausahakul et al., 2008). However, viral elements such as domains, motifs, and amino acid residues, involved in the pathogenesis of street viruses have yet to be identified. One reason for this is that most studies were performed with fixed strains although the genetic similarity between street and fixed strains is not very high.

The number of *N*-glycosylation sites in the G protein might be related to the difference between street and fixed viruses. Several fixed viruses have three or four potential glycosylation sites on the G protein, and most have two sequons, Asn at positions 37 and 319 (Asn^{37} and Asn^{319}) (Morimoto et al., 1992). Recently, Ming et al. (2009) mentioned that the Chinese street virus HN10 has only two sequons at Asn^{37} and Asn^{319}, and that the sequon at Asn^{247}, which is present in many fixed viruses, is absent in other street viruses as well. Interestingly, in the West Nile virus (WNV), *N*-glycosylation of the envelope (E) protein is responsible for neuroinvasiveness and growth efficiency in cells (Beasley et al., 2005; Shirato et al., 2004). However, there is as yet no direct evidence for the impact of *N*-glycosylation on the rabies virus' biological activity.

Parasites, including viruses, are attenuated after serial passaging in cell cultures (Ebert, 1998). Interestingly, several attenuated fixed rabies viruses regained neurovirulence (killing adult mice after i.c. inoculations) after a few passages in murine or human neuroblastoma cell lines (Clark, 1978). Therefore, since we suspected that the neurovirulence of rabies viruses is maintained during serial passaging in mouse neuroblastoma NA cells, in this study we have attempted to establish an attenuated street virus variant by serial passage of strain 1088 in NA cells in order to identify the viral element(s) responsible for the pathogenecity of street viruses other than neurovirulence. We obtained an NA cell-adapted variant, which was attenuated in mice after i.m. but not i.c. inoculations. This variant had only seven nucleotide substitutions compared to the parental strain, one of which led to additional *N*-glycosylation of the G protein. Furthermore, we established a cloned variant, the G protein of which possessed the single mutation. This variant was also adapted to NA cells and attenuated in mice after i.m. inoculations. Hence, we propose that the additional *N*-glycosylation of the G protein is likely related to the adaptation to NA cells and attenuation of the pathogenicity of street rabies viruses.

2. Materials and methods

2.1. Cell and virus

Mouse neuroblastoma C1300 (NA) cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Street rabies virus 1088, a strain isolated from a woodchuck in the Centers for Disease Control in Atlanta, USA was obtained from the Yale Arbovirus Unit, Yale University (Mifune et al., 1979). The strain was passaged in the brains of suckling mice only twice since the original isolation. The supernatant of the 10% brain homogenate was used.

2.2. Serial passage

Monolayered NA cells in 6-well plates, T-75 flasks, or T-25 flasks were used for the serial passage of strain 1088. Until the 24th passage, each virus stock was diluted 10-fold with EMEM containing 2% FCS and inoculated into NA cells at 150 μl per well in 6-well plates, 500 μl in T-25 flasks, or 1 ml in T-75 flasks. After the 25th passage, the virus was diluted 100- or 1000-fold with EMEM containing 2% FCS. After adsorption for 1 h at 37 °C, the inoculum was removed, and Dulbecco's modified Eagle's medium (DMEM) containing 5% FCS was added at 2.5 ml per well in 6-well plates, 5 ml in T-25 flasks, or 15 ml in T-75 flasks. The inoculated cells were incubated at 37 °C for several days. When about 50% of the cells were detached, the medium was collected and centrifuged at 2000 × *g* and 4 °C to remove debris. The supernatant was collected and stored at −80 °C until used.

2.3. Virus titration

The virus was titrated by a focus assay on confluent monolayers of NA cells in 24-well plates. Serial 10-fold dilutions of virus were made with EMEM containing 2% FCS and then 100 μl of each dilution was inoculated into the cells in quadruplicate. After 1 h at 37 °C, the cells were washed twice with Hanks' balanced salt solution. Subsequently, the cells were overlaid with EMEM containing 5% FCS and 1% (w/v) methylcellulose (catalog no. M0512, SIGMA) and then incubated at 37 °C. At 4 days post-inoculation (p.i.), the cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS). Subsequently, the cells were stained with FITC Anti-Rabies Monoclonal Globulin (Fujirebio Diagnostics, Inc.). Viral foci were counted under an inverted fluorescent microscope, and the viral titer was expressed as focus-forming units (FFU).

2.4. Nucleotide sequence analysis

Viral RNA was isolated from a virus stock or cells using Trizol reagent (Invitrogen). Subsequently, cDNA was synthesized using random hexamers and SuperScript III reverse transcriptase (Invitrogen). PCR was performed with Ex-Taq (Takara Bio inc., Japan). The 3′ and 5′ ends of the viral genome were amplified from total RNA of virus-inoculated cells by 5′-RACE (rapid amplification of cDNA ends) using the SMART RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions. The amplified DNA fragment was purified using a Nucleospin Extract II kit (Macherey-Nagel) and then the DNA sequence was determined using BigDye Terminator ver. 3.1 and the ABI 3130 Genetic Analyzer

(Applied Biosystems). The sequences obtained were analyzed with GENETYX-MAC software ver. 15 (Genetyx corp., Japan). Detailed information of primers used is available from the authors on request. The complete genome sequence of strain 1088 determined in this study has been deposited in DDBJ/EMBL/GenBank (GenBank ID: AB645847).

2.5. Virus cloning by limiting dilution

The *N*-glycosylation variant of 1088 was cloned from the stock of the fourth passage by limiting dilution as follows. The stock was diluted to 2.5 FFU/ml and 200 μl of the diluted solution was added to monolayered NA cells in 12-well plates (0.5 FFU/well). After adsorption for 1 h, the inocula were removed and 3 ml of DMEM containing 5% FCS was added to the cells. The inoculated cells were incubated at 37 °C. At 11 days p.i., the culture medium was collected and stored at −80 °C. Simultaneously, the cells were processed for Western blotting as described below. After the mobility of the G proteins of the clones was checked by Western blotting, the positive clone was propagated twice in NA cells and then the entire genome was sequenced as described above. Finally, we chose one clone, named 1088-N4#14.

2.6. Plasmid construction

An expression vector for the 1088 (N0) G or variant (1088-N4#14 or 1088-N30) G was constructed as follows. Each G gene was amplified from the corresponding cDNA by PCR with KOD Plus ver. 2 (Toyobo, Japan) using the primers EcoRI-1088-Gf (5′-AACCGGAATTCCACCATGGTTCCTCAGGCTCTT-3′; EcoRI site is underlined) and XbaI-1088-Gr (5′-AAAGCTCTAGATTCACAGACTGGTCTCACC-3′; XbaI site is underlined). The DNA fragment was cloned into the pCI vector (Promega) after digestion with EcoRI and XbaI. Newly constructed plasmids were sequenced for verification.

2.7. Tunicamycin treatment

NA cells were inoculated with each virus at a multiplicity of infection (MOI) of 1 and then incubated in the absence or presence (1 μg/ml) of tunicamycin (Sigma–Aldrich). At 28 h p.i., the cells were processed for Western blotting as described below.

The plasmid encoding the G protein (0.5 μg/well) was transfected into NA cells in 24-well plates using TransIT-Neural reagent (Mirus) according to the manufacturer's instructions. At 4 h post-transfection, the medium was replaced with EMEM containing 10% FCS with or without 1 μg/ml tunicamycin. After additional incubation for 20 h, the transfected cells were processed for Western blotting.

2.8. Western blotting

Cells were lysed with a lysis buffer [50 mM Tris–HCl (pH 8.0), 1% NP-40 and 150 mM NaCl], and then mixed with the same amount of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [125 mM Tris–HCl (pH 6.8), 4% SDS, 10% (w/v) sucrose, 10% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue]. After incubation at 95 °C for 5 min, proteins in the samples were separated by SDS-PAGE in a 10% gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane (0.45 μm; Millipore) for analysis by Western blotting. After blocking with PBS containing 5% skim milk and 0.1% Tween-20, the membrane was incubated with the anti-G mouse monoclonal antibody (MAb) 15-13 (Luo et al., 1998), anti-N mouse MAb 8-1 (Minamoto et al., 1994), or anti-β-actin mouse MAb G043 (Applied Biological Materials Inc., Canada) in PBS containing 2.5% skim milk and 0.1% Tween-20. After being washed with PBS containing 0.1% Tween-20, the membrane was incubated with peroxidase-conjugated anti-mouse IgG (Cappel) in PBS containing 2.5% skim milk and 0.1% Tween-20. Protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

2.9. Virus growth in NA cells

Monolayered NA cells in 24-well plates were inoculated with each virus at an MOI of 0.01 or 1. After adsorption for 1 h, the cells were washed twice with Hanks' balanced salt solution and resupplied with 1 ml of EMEM containing 5% FCS per well. The cells were incubated at 37 °C and samples of the culture medium were harvested at 24, 48, 72, and 96 h p.i. Each sample was centrifuged at 800 × *g* for 10 min, and its supernatant was stored at −80 °C. The viral titer in a sample was determined in NA cells as described above. In addition, the remaining cells were processed for Western blotting as described above.

2.10. Pathogenicity of viruses in adult mice

Six-week-old female ddY mice (Kyudo Co. Ltd., Japan) were used. Serial 10-fold dilutions of each virus were made with PBS containing 2% FCS. Subsequently, 0.03 ml of each dilution was injected i.c. into groups of five mice. Meanwhile, 0.05 ml of each dilution was injected by i.m. (right hindlimb of mice). The inoculated mice were weighted every day and observed for 28 or 42 days after the i.c. or i.m. injection, respectively. The 50% lethal dose (LD_{50}) was calculated by the method of Reed and Müench (1938). All animal experiments were performed with the approval of the ethics committee of Oita University.

2.11. Immunohistochemistry

Fifteen ddY mice (6-week-old, female) per group were inoculated with 10^6 FFU of each strain. At 5, 8 and 11 days p.i., five mice per group were sacrificed and their sera, tissues and organs were sampled. The tissues and organs were fixed with a 10% Formalin Neutral Buffer Solution (Wako, Japan). Immunohistochemistry with brain sections embedded in paraffin was performed using the anti-P rabbit serum (Shoji et al., 2004) as described previously (Kojima et al., 2010).

2.12. Rapid fluorescent focus inhibition tests

Viral neutralizing antibodies (VNA) against the rabies virus were determined by rapid fluorescent focus inhibition tests (RFFIT) as described previously (Shiota et al., 2009; Smith et al., 1973). VNA titers were represented as international units per ml (IU/ml), which was calculated by comparison with WHO international standards [RAI: Anti-rabies Immunoglobulin, human, National Institute for Biological Standards & Control (NIBSC)].

2.13. Virus internalization assay

Monolayered NA cells in 24-well plates were incubated with approx 120 FFUs of each virus per well for several time periods (10, 20, 40, 60, and 80 min) at 37 °C. The cells were then washed twice with Hanks' balanced salt solution and overlaid with EMEM containing 5% FCS and 1% (w/v) methylcellulose. The cells were incubated at 37 °C for 4 days, and viral foci were visualized as described above. The efficiency with which each virus was internalized is expressed as the relative number of foci, with the number at 80 min as 100%.