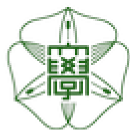
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2 **Title page**

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Author contributions

Zhouoxing Wei; Preparation of antigens and perform all experimental work. Analysis of data, and writing this paper, assembly of data, and writing manuscript.

Kenta Shimizu, Yoshimi Tsuda, Jiro Arikawa; Provided expert knowledge on hantaviruses, design of experiment and wrote manuscript.

Kumpei Nishigami; Cloning and expression of Asama virus N protein.

Lishanta Granathne, Nishanta Nanayakkara; Diagnose of CKD and collection of patient and control sera.

Yomani Sarathukumara, Devinda S. Muthusinghe, Chandika D. Gamage, Sithumini M.W. Lokupathirage; Collection of human sera from patients healthy.

Fuka Kikuchi, Keiko Tanaka-Taya, Motoi Suzuki, Shigeru Morikawa; Preparation of Altai virus and Seewis virus cDNA and amplification of their coding region of N proteins.

Satoru Arai, Kumiko Yoshimatsu; Conception of this study and design of experiment, analysis of data, and editing this paper.

Abstract

The infectivity of shrew-borne hantaviruses to humans is still unclear because of the lack of a serodiagnosis method for these viruses. In this study, we prepared recombinant nucleocapsid (rN) proteins of Seewis orthohantavirus, Altai orthohantavirus (ALTV), Thottapalayam thottimvirus (TPMV) and Asama orthohantavirus. Using monospecific rabbit sera, no antigenic cross-reactivities were observed. In a serosurvey of 104 samples from renal patients and 271 samples from heathy controls from Sri Lanka, one patient and two healthy control sera reacted with rN proteins of ALTV and TPMV, respectively. The novel assays should be applied to investigate potential infectivity of shrew-borne hantaviruses to humans.

Running title: Serological detection for shrew-borne hantavirus

Hantaviruses are enveloped single-stranded negative-sense RNA viruses that belong to the family *Hantaviridae* of the order *Bunyavirales*. Some viruses are responsible for two fatal rodent-borne zoonotic diseases in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). The nucleocapsid (N) protein not only plays an essential role in viral replication and assembly [16] but is also the main target for immune response and is used as a diagnostic antigen for hantavirus infection [1, 6, 25, 30].

Various shrew-borne hantaviruses have been reported in many countries [28]. Thottapalayam thottimvirus (TPMV) was the first isolated hantavirus. It was isolated from *Suncus murinus* (musk shrew or Asian house shrew) captured in southern India in 1964 [5]. Full-genome analysis of TPMV showed an early evolutionary divergence

from rodent-borne hantaviruses [26, 27], and very low or no antigenic cross-reactivity has been observed between TPMV and other rodent-borne hantaviruses [7, 19]. In serological screening in Asian countries, only one human seropositive case of TPMV has been found in Thailand [18]. However, a conclusion about the infectivity and pathogenicity of TPMV to humans cannot be made because of the small number of screened cases. On the other hand, TPMV-seropositive and RNA-positive Asian house shrews have been found in Vietnam, Indonesia, India and China [5, 9, 12, 15, 19].

Seewis orthohantavirus (SWSV) is widely distributed in European and Siberian Russia, and several species of *Sorex* shrews could be its natural hosts [13]. Ling et al. investigated a truncated SWSV N antigen since the N-terminal 120 amino acids have been shown to be highly antigenic. They reported a low frequency of human SWSV infection and low degrees of cross-reactivity between SWSV and other types of hantaviruses. There is no evidence showing pathogenicity of SWSV to humans [13].

Phylogenetic analysis of SWSV showed well-resolved lineages organized by geographic origin [14]. Eurasian common shrews (*S. araneus*) captured in Hungary and Russia showed a highly divergent hantavirus lineage, and comparison of 300-nucleotide regions of L segments indicated that a distinct hantavirus was being maintained, and it was proved to be Altai orthohantavirus (ALTV). ALTV is distributed widely in Russia, Mongolia, and Europe and is carried by various species of shrews including *S. araneus, S. caecutiens, S. minutissimus*, and *S. roboratus* [11]. However, antigenic characterization of ALTV has not been reported yet.

Phylogenetic analysis has shown that Asama orthohantavirus (ASAV) is closer related to SWSV than to ALTV and TPMV [4]. ASAV is carried by Japanese shrew

moles (*Urotrichus talpoides*) and these animals are endemic to Japan, suggesting that the distribution of ASAV is limited to Japan.

Genetic analyses indicated that shrew-borne hantaviruses are variable [3, 10]. Despite numerous genetic reports, antigenic relationships among various shrew-borne viruses have not been reported because serological detection methods have been developed for only a few types of shrew-borne hantaviruses [19, 25].

In this study, we prepared recombinant N (rN) protein expression plasmids of ALTV (from *S. tundrensis*), SWSV (from *Sorex tundrensis*), TPMV and ASAV (from *Urotrichus talpoides*) for serological diagnostic antigens. The coding region of the N protein of ASAV (EU929070) was previously prepared [25]. An expression vector for TPMV N protein (AY526097) with amino acid exchanges for monoclonal antibody E5/G6 binding (TPMV-mu; Fig. 1A) was previously prepared [23]. The coding regions of N proteins of ALTV (MT544323) and SWSV (MT544324) from shrews captured in the Khovsgol region of Mongolia in August 2010 were amplified by PCR as described previously [31]. Coding information of the N proteins of SWSV, ALTV, and ASAV was inserted into a mammalian expression vector, pCAGGS/MCS [17]. Briefly, the N protein coding regions of ASAV, ALTV and SWSV were amplified by PCR using the following primers: AGGAATTCATGGCAACATTGAGGACATCC and CGTCTCGAGTTACAGCTTGAGAGGATCCATGTTTGAAATC for ASAV, TATGAATTCATGGCAGATATAAAGCAGGG and TTTCTCGAGTTACAGCTTTAATGGTTCCT for ALTV, CACGAATTCATGGAGGATATCAAACAGTT and TTTCTCGAGTCACAGCTTCATTGGCTCCA for SWSV. The PCR products were purified and digested with EcoRI (Takara, Kusatsu, Japan) and XhoI (Takara). The

1 fragments were inserted into the mammalian expression plasmid vector pCAGGS/MCS
2 and digested with EcoRI and XhoI. After confirming the sequences of constructs,
3 plasmid DNAs of pC-ASAV-NP, pC-TPMV-NP, pC-ALTV-NP and pC-SWSV-NP
4 were prepared by using a QIAfilter plasmid Maxiprep Kit (Qiagen, Hilden, Germany).
5 Also, the coding regions of N proteins of SWSV and ALTV were inserted into the
6 expression vector pET43.1 (Novagen, Merck Millipore, Burlington MA, USA) and
7 digested with EcoRI and XhoI for expression of N proteins fused with Nus and
8 histidine-tagged proteins on the N- terminal region. These constructs, pET43.1-ALTV-
9 NP and pET43.1-SWSV-NP, were introduced into *E. coli* strain BL21 (DE3)
10 (Novagen). Recombinant proteins were synthesized and then purified using HisTrap HP
11 columns (Amersham, Piscataway, NJ, USA) according to the manufacturer's
12 instructions. Molecular weights of purified antigens were evaluated by a Western blot
13 assay by using Nus•Tag™ Monoclonal Antibody (Novagen) (data not shown). Rabbit
14 antisera to Nus-tagged rN proteins of ALTV and SWSV were prepared by Sigma
15 Aldrich Technical Service Japan. Rabbit antisera to TPMV rN proteins and mice sera
16 raised against TPMV were previously prepared [19]. The mouse monoclonal antibody
17 E5/G6 to the N protein of Hantaan orthohantavirus was used [18, 29]. Interestingly, the
18 N protein of ASAV had an epitope sequence of E5/G6 (Fig. 1A). Therefore, we used
19 the E5/G6 monoclonal antibody to detect rN proteins of TPMV-mu and ASAV.
20 In order to confirm the expression of rN proteins in mammalian cells, pC-ASAV-NP,
21 pC-TPMV-NP-Mu, pC-ALTV-NP and pC-SWSV-NP were transfected to Vero E6 cells
22 by using TransIT-LT1 transfection reagent (Mirus, Madison, WI, USA), and rN proteins
23 were detected by an indirect immunofluorescence antibody assay (IFA) (Fig. 1B). Alexa
24 Fluor 488 goat anti-mouse IgG antibody (Thermo Fisher Scientific, Life Technologies,

Waltham, MA, USA) was used as a secondary antibody for the E5/G6 monoclonal antibody. Alexa Fluor 488 Protein A (Thermo Fisher Scientific) was used to detect antibodies in shrew, rabbit and human sera.

The rN protein of TPMV-mu had been modified for binding with E5/G6. E5/G6 showed fluorescence in the cytoplasm of pC-ASAV-NP and pC-TPMV-mu-NP-transfected cells (Fig. 1B). Similarly, anti-rN of ALTV and SWSV immune rabbit sera showed fluorescence in cells expressing the homologous antigens. As shown in Fig. 1B, mock-transfected cells and unimmunized rabbit sera showed no specific fluorescence. These results indicated that rN proteins of ASAV, TPMV-mu, ALTV, and SWSV were successfully expressed in Vero E6 cells. Recombinant N protein of ASAV was only detected by E5/G6 and showed no-cross reactivity to antisera to TPMV-mu, ALTV, and SWSV. These results indicated highly divergent antigenic characteristics of N proteins of the four shrew-borne hantaviruses.

Plasmids pC-ASAV-NP, pC-TPMV-NP, pC-ALTV-NP and pC-SWSV-NP were transfected to HEK293T cells. At 48 hrs after transfection, the cells were collected and lysed for Western blot assays. Immune rabbit sera and E5/G6 were used as first antibodies, and the binding antibodies were detected by using horseradish peroxidase (HRP)-conjugated protein A (Prozyme, San Leandro, CA, USA) and ECL Prime Western Blotting Detection Reagent (Amersham). No cross-reactivities of immune sera among ASAV, TPMV-mu, ALTV, and SWSV were also confirmed by Western blot assays. As shown in Fig. 1C, molecular weights of detected rN proteins were around 50 KDa, which were close to the estimated sizes of N. Since degraded products were also detected, it is thought that there are sites that are easily cleaved. E5/G6 detected both rN proteins of ASAV and TPMV-mu expressed in HEK293T cells. On the other hand, anti-

TPMV, ALTV, and SWSV immune sera detected only the homologous rN antigen. The use of a combination of two different assays, IFA using Vero E6 cells and Western blot assay using HEK293T cells, is expected to be effective for excluding reactivities against cellular components of Vero E6 cells observed in an IFA assay. Finally, sera that responded to both tests, Vero E6 cell-based IFA and HEK293T-based Western blot assay, were considered as positive.

Sera from HFRS patients or febrile patients were used to confirm cross-reactivities of the four shrew-borne hantavirus antigens to pathogenic hantaviruses [2, 20]. Four anti-Seoul orthohantavirus serum samples, three anti-Hantaan orthohantavirus serum samples, two anti-Puumala orthohantavirus-positive serum samples and one anti-Thailand orthohantavirus (THAIV)-positive serum sample, showed no reactivities to rN antigens of TPMV-mu, ASAV, ALTV, and SWSV. None of monoclonal antibodies against TPMV N [25] cross-reacted with ASAV, ALTV, and SWSV antigens (data not shown). No reactivities to the four shrew-borne hantavirus antigens were observed with six sera from healthy donors used in previous research [2]. These results suggested low or no cross-reactivities among ASAV, TPMV, ALTV, SWSV and rodent-borne hantaviruses. Although it was not fully validated, the specificity of these serological tests has been demonstrated to some extent. However, the sensitivity of the tests cannot be evaluated due to the lack of definite positive serum specimens. Further studies are needed to establish this serological screening system.

Recently, we reported almost 50% seroprevalence against THAIV among patients with chronic kidney disease of unknown etiology (CKDu) in Sri Lanka [8, 32]. THAIV is not considered to be a causative agent for HFRS or HPS [20, 22], but a significant relationship between seropositivity to THAIV and renal disease was observed in this

area [24]. In this study, we tried to clarify the association between shrew-borne hantavirus infection and renal diseases. Sera from 104 renal disease patients and 275 healthy controls collected from 2016 to 2018 in Girandrukotte, Sri Lanka were used [8, 24]. Ethical approval for this study was obtained from the Institutional Ethical Review Committee, Faculty of Medicine, University of Peradeniya, Sri Lanka (2016/EC/64) and the Ethical Review Committee of the Graduate School of Medicine, Hokkaido University, Japan (M17-023). As shown in Fig. 2A, seropositivity rates were very low and no significant difference between patients and healthy controls was found. These results indicated that there was no association between seropositivity to shrew-borne hantaviruses and renal disease. However, we found one serum specimen that was positive for ALTV antigen (#1) from patients and two serum specimens that were positive for TPMV antigen (#2 and #3) from healthy controls. Western blot profiles of these sera are shown in Fig. 2B. In IFA, serum specimen #1, #2 and #3 also showed specific reactions to ALTV and TPMV (data not shown). Interestingly, specimens #1 and #3 were also THAIV-seropositive in previous studies [24, 32]. Because of the lack of antigenic cross-reactivities of N antigen among TPMV, ALTV, and THAIV, transmission of TPMV or ALTV from shrews and transmission of THAIV from rodents might have occurred independently in this area. For further antigenic comparison, focus reduction neutralization (FRNT) assays were carried out as previously described [15]. Although the anti-TPMV immune serum used as a positive control showed a high FRNT titer of over 160 [19], neither #2 nor #3 showed a significant focus reduction (<20). These results suggested that a TPMV-related but serologically distinct virus might have infected humans in Girandurukotte, Sri Lanka. Unfortunately, isolated ALTV was not available, and we could not perform an FRNT assay for ALTV.

The results suggested the potential infectivity of shrew-borne hantaviruses to humans. However, there is still no strong evidence showing that these viruses caused renal disease in humans. In Sri Lanka, TPMV or ALTV- positive shrews have not been reported yet. However, Sri Lanka is a habitat of many species of shrews. It is necessary to find reservoir shrews as a source of hantavirus infection to humans. Recently, Qi et al. suggested that the N protein of Imjin virus shows no antigenic cross-reactivities to rodent-borne hantavirus, and they also suggested Imjin or Imjin-like virus infection in humans in China [21]. Their results also suggested that potential human infection of shrew-borne virus has occurred in various countries.

In this study, by using a novel serological diagnostic method, we found the first seropositive human case to ALTV antigen. Although most of the shrew-borne hantaviruses have not been isolated, the serodiagnostic method that we have developed can be used without isolated viruses. Therefore, this method is useful for further serological investigations of shrew-borne hantaviruses.

Compliance with ethical standards

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Conflict of interest: The authors declare that there is no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants

1 were in accordance with the ethical standards of the institutional and/or national
2 research committee and with the 1964 Helsinki declaration and its later amendments or
3 comparable ethical standards.
4 ***Informed consent:*** Written informed consent was obtained from all individual
5 participants included in the study.
6
7 **Figure legends**
8 Fig.1. Expression of rN proteins of ASAV, TPMV, ALTV and SWSV.
9 A: Epitope sequence of E5/G6 in TPMV and ASAV. The deduced amino acid sequence
10 of the E5/G6 epitope of ASAV is close to that of Hantaan virus. On the other hand, wild-
11 type N of TPMV lacked binding with E5/G6. The rN of a TPMV mutant (TPMV-mu)
12 had four amino acid substitutions to enable binding with E5/G6 [19, 23]. Corresponding
13 regions of ALTV and SWSV are also shown.
14 B: Vero E6 cells expressing rN on 24-well glass slides were fixed with acetone.
15 Monoclonal antibody E5/G6 and rabbit antisera to rN of TPMV, ALTV and SWSV were
16 used for detection of rN of ASAV, TPMV, ALTV and SWSV, respectively. Cells
17 transfected with pCAGGS/MCS plasmid were used as negative control cells (Mock).
18 C: HEK293T cells transfected with rN were lysed and used as an antigen for a Western
19 blot assay, and the same antibodies as those in B were used for detection of rN proteins.
20
21 Fig. 2. Serological screening of human sera by using rN of ASAV, TPMV-mu, ALTV,
22 and SWSV.
23 A: Numbers of sera that are antibody positive to ASAV, TPMV-mu, ALTV, and SWSV
24 were confirmed by two tests, IFA and Western blot assay, at a serum dilution of 1:100.

Number of antibody positive sera to THAIV was confirmed by two IFA tests by using rN of THAIV-expressing cells and THAIV-infected cells as previously described [24] . We found one seropositive for ALTV antigen (#1) from patients and two seropositives for TPMV-mu antigen (#2 and #3) from healthy controls.

B: Western blot patterns of human sera #1, 2, and 3 to rN antigens of ASAV, TPMV, ALTV, and SWSV.

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