Yoshii, K., Konno, A., Goto, A., Nio, J., Obara, M., Ueki, T., Hayasaka, D., Mizutani, T., Kariwa, H. & Takashima, I. (2004). Single point mutation in tick-borne encephalitis virus prM protein induces a reduction of virus particle secretion. *J Gen Virol* **85**, 3049–3058.

Yoshii, K., Hayasaka, D., Goto, A., Kawakami, K., Kariwa, H. & Takashima, I. (2005). Packaging the replicon RNA of the Far-Eastern subtype of tick-borne encephalitis virus into single-round infectious particles; development of a heterologous gene delivery system. *Vaccine* **23**, 3946–3956.

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BRIEF REPORT

Development of a serotyping ELISA system for Thailand virus infection

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Abstract To distinguish Thailand virus infection from infections with other hantaviruses, we established an ELISA serotyping system using a truncated nucleocapsid protein of Thailand virus lacking 49 amino acids at the N-terminus. In evaluations using patient and rodent sera, Thailand virus infection was readily distinguished from Hantaan and Seoul virus infections. Therefore, this ELISA system is an effective alternative to neutralization tests.

Hemorrhagic fever with renal syndrome (HFRS) is a rodent-borne viral zoonosis caused by viruses belonging to the genus *Hantavirus*, family *Bunyaviridae* [1]. Four hantaviruses are currently known to be causative agents of HFRS: Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava–Belgrade virus (DOBV), and Puumala virus (PUUV). Hantaviruses are considered to be closely associated with their rodent hosts due to a coevolution process [2, 3].

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Thailand virus (THAIV) was isolated from a greater bandicoot rat (*Bandicota indica*) captured in Thailand [4]. THAIV has a distinct focus reduction neutralization test (FRNT) pattern [5] and was shown to belong to a distinct lineage by phylogenetic analysis [6]. Recently, we reported the first HFRS case related to THAIV [7]. A seroepidemiological study revealed the existence of hantavirus infection among patients in Thailand with leptospirosis-like symptoms [7, 8]. Only a few cases of THAIV infection have been reported to date, but we believe that THAIV causes HFRS-like disease in Southeast Asia. Due to its immunological cross-reactivity with HTNV, SEOV, and DOBV, the FRNT is required to differentiate between hantavirus serotypes [9, 10]. However, the FRNT requires specific technical skill and a biosafety laboratory for handling viruses. To overcome these limitations, we developed a serotyping ELISA system using the truncated recombinant nucleocapsid proteins (NPs) of HTNV, SEOV, and DOBV [9]. The hantavirus NPs possess immunodominant, linear, and cross-reactive epitopes within their N-terminal 100 amino acids (aa) [9, 11, 12]. By removing 49 aa from the N-terminus, serotyping antigens that retained serotype-specific, multimerization-dependent epitopes in the C-terminal half of the NPs were prepared [12, 13]. In this study, the application of the serotyping ELISA system was expanded to the diagnosis of THAIV infection.

THAIV strain thai749 was kindly supplied by Dr. P. W. Lee (WHO Collaborating Center for Virus Research, Korea). The virus was propagated in the E6 clone of Vero cells (ATCC C1008, CRL 1586). Recombinant baculoviruses (Autographa californica nuclear polyhedrosis virus) containing regions encoding the whole and truncated NPs of hantaviruses (HTNV, SEOV, and THAIV) were propagated in High Five cells, as described previously [9]. The cDNA of THAIV strain thai749 was prepared as described previously

[14]. A portion of the gene encoding the entire THAIV NP was amplified using the polymerase chain reaction (PCR) with the primers Bam_THLS46F (5′-*GGATCC*ATGGCAA CTATGGAAGAG-3′) and Bam_THLS1344R (5′-TGTGG*G GATCC*TAGAGTTTTAA-3′; the *Bam*HI sites are shown in italics) and that of aa 50–429 of THAIV NP was amplified by PCR with primers Bam_THLS193F (5′-AC*GGATCC*ATG GTGGCTGCATCAAT-3′) and Bam_THLS1344R. The amplified DNA was subcloned into the *Bam*HI site of the donor plasmid pFAST-Bac1 and then expressed using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA). The indirect immunofluorescent antibody (IFA) assay was carried out as described previously [15]. As shown in Table 1, the whole rNP of THAIV exhibited the same reactivity pattern to monoclonal antibodies (MAbs) as the corresponding authentic viral antigens, which showed that the rNP was well conserved. The MAb E5/G6, which recognizes a common epitope of hantavirus NPs [16], could detect full-length and truncated rNP of THAIV, demonstrating that MAb E5/G6 is useful as a capture antibody for THAIV antigens [9]. We can provide E5/G6 MAb upon request. HTNV-specific (C24B4 and BDO1) and SEOV-specific (DCO3) MAbs could not detect the THAIV rNPs. As an epitope of MAb ECO2 is localized between aa residues 1 and 33 of the N-terminus [12], the truncated NP of THAIV possess reactivity to MAb ECO1, but the reactivity was lower than that obtained with other truncated rNPs of HTNV and SEOV. The results for THAIV antigens shown in Table 1 were consistent with those reported previously for HTNV, SEOV, and DOBV [9].

A total of 96-well plates were coated for 1 h with MAb E5/G6 in PBS as a capture antibody, and then ELISA was performed as described previously [9]. Sera from HFRS patients previously diagnosed as being infected with HTNV, SEOV, and THAIV were used, and two human sera confirmed to contain no antibodies to any hantavirus by ELISA, IFA and Western blot were used as negative controls [7, 9, 17]. Figure 1a shows reaction patterns of patient sera showing OD values greater than 0.7 against whole NP from the homologous virus. All three whole rNPs reacted well with sera from patients infected with, HTNV, SEOV, or THAIV (Fig. 1a, left). In contrast, each truncated rNP reacted strongly with homologous sera, but reacted either not at all or with much lower intensity with heterologous sera (Fig. 1a, right). Figure 1b shows the effectiveness of the test for serotyping patient sera infected with HTNV, SEOV, or THAIV. The sera shown in Fig. 1a were re-examined and included in Fig. 1b (marked with asterisk). In Fig. 1b, upper panel, ELISA OD values of HTNV, SEOV, and THAIV patient sera to homologous or heterologous whole rNPs were plotted with different markers. As shown, the regression lines of markers for HTNV and SEOV patients crossed with each combination of antigens. Further markers for paired sera of THAIV patient were plotted close to the regression lines. Therefore, whole rNPs were not applicable for serotyping of THAIV infection. On the other hand, using the truncated rNPs, the serotypes of the infecting viruses could be distinguished clearly (Fig. 1b, lower panel). However, the ELISA OD value using truncated NP antigen was lower in some cases than that obtained with whole NPs. Therefore, the sensitivity of the ELISA using truncated rNPs might be lower than with whole rNPs. These results indicate that whole rNP antigens are effective for detecting anti-hantavirus antibodies and that the series of truncated rNPs (aa 50–429) is effective for serotyping.

Table 1 Antigenic profiling of recombinant antigens using MAbs directed to hantavirus NPs in IFA

MAbs	IFA antigens								
	Vero E6 cells infected with			High Five cells infected with recombinant baculovirus expressing the whole N protein from:			High Five cells infected with recombinant baculovirus expressing a truncated N protein (aa 50–429) from:		
	HTNV	SEOV	THAIV	HTNV	SEOV	THAIV	HTNV	SEOV	THAIV
Cross-reactive clones									
ECO2	+	+	+	+	+	+	−	−	−
ECO1	+	+	+	+	+	+	+	+	±
E5/G6	+	+	+	+	+	+	+	+	+
HTNV-specific clones									
C24B4	+	−	−	+	−	−	+	−	−
BDO1	+	−	−	+	−	−	+	−	−
SEOV-specific clone									
DCO3	−	+	−	−	+	−	−	+	−

IFA profiles against Vero E6 cells infected with HTNV, SEOV, and THAIV were reported previously [7], and IFA profiles against High Five cells infected with recombinant baculovirus expressing the whole N protein and a truncated N protein (aa 50–429) from HTNV and SEOV were reported previously [9]

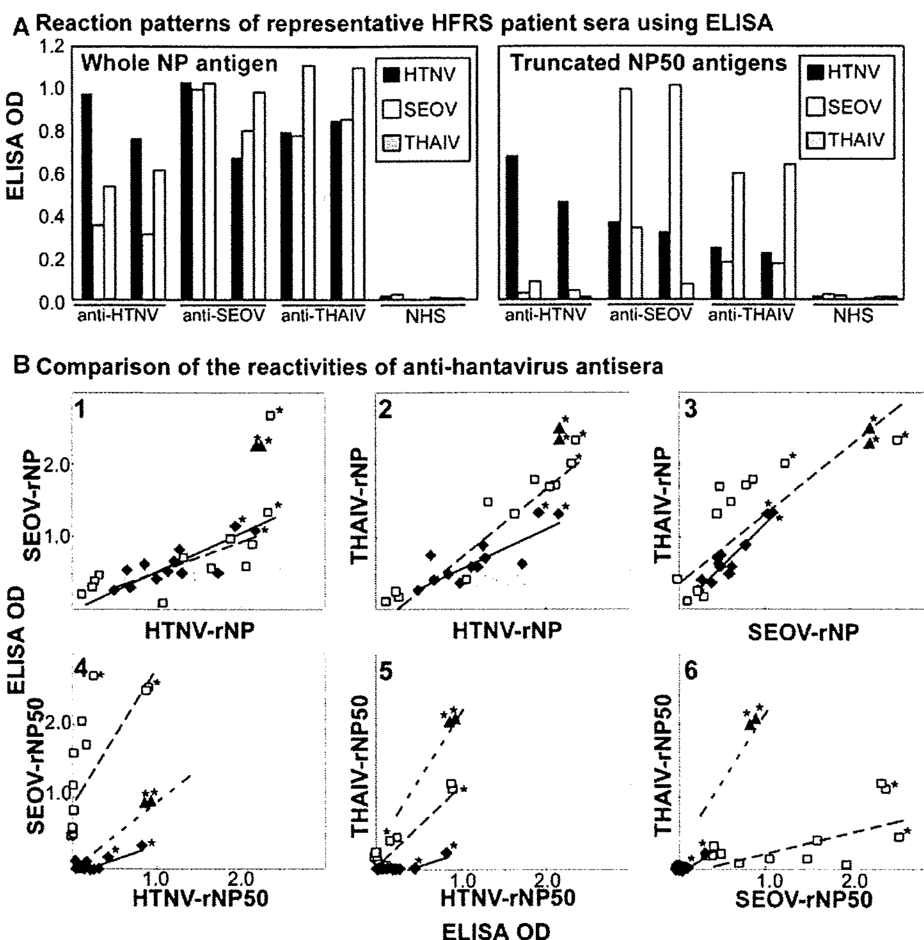

Fig. 1 Reactivities of representative and groups of patient sera against recombinant and truncated NP antigens in ELISA. **a** Reaction patterns of representative HFRS patient sera. Anti-HTNV patient sera were obtained from China. Anti-SEOV patient sera were obtained from Japan to Korea, associated with rat-borne disease outbreaks in laboratories. Two anti-THAIV serum samples were obtained from the same patient: one from the acute phase and another obtained 1 year after the first sample. NHS is a human serum obtained from a single individual confirmed to be negative for hantavirus-specific antibodies, obtained from Japan. The serotypes of infecting viruses were determined by FRNT. The ELISA OD values of sera against whole rNP antigens (*left panel*) and truncated NP antigens for aa 50–429 (*right panel*) of HTNV (*black bars*), SEOV (*white bars*), and THAIV (*gray bars*) are shown. **b** Comparison of the reactivities of anti-hantavirus antisera with the recombinant antigens used in this study. The horizontal and vertical axes show the ELISA ODs for sera from HTNV-infected patients (*diamonds*), SEOV-infected patients (*square*), and a THAIV-infected patient (*triangle*) for each antigen. A total of 23 serum samples were used to assess our serotyping ELISA system: 11 anti-HTNV samples obtained from China, 10 anti-SEOV samples obtained from Japan to Korea, associated with rat-borne outbreaks in laboratories, and two anti-THAIV samples were the same sera shown in panel A. The serotypes were determined by FRNT [7, 9, 17]. ELISA ODs were compared as follows: 1, HTNV-rNP versus SEOV-rNP; 2, HTNV-rNP versus THAIV-rNP; 3, SEOV-rNP versus THAIV-rNP; 4, HTNV-rNP50 versus SEOV-rNP50; 5, HTNV-rNP50 versus THAIV-rNP50; 6, SEOV-rNP50 versus THAIV-rNP50. The *lines* show linear regressions for each group of sera: *solid lines*, sera from HTNV-infected patients; *dashed lines*, sera from SEOV-infected patients; *broken lines*, sera from a THAIV-infected patient

To demonstrate the applicability of the serotyping ELISA, antisera derived from several rodents were prepared. Two WKAH/hkm rats and four Slc:ICR mice (SLC, Hamamatsu, Japan) were inoculated intraperitoneally with 10^4 FFU of THAIV strain thai749 per animal, and serum was collected after 5 weeks. All of the animals were treated in accordance with the laboratory animal control guidelines of our institute, which conform to those of the U. S. National Institutes of Health. All animal experiments were carried out in a BSL3 facility. Three serum samples from greater bandicoot rats from Thailand were obtained. Two of them were confirmed to be positive for anti-hantavirus antibody by IFA, ELISA, and FRNT in a previous study [7]. The third serum sample was used as a negative control. For the rat and greater bandicoot rat serum samples, bound antibodies were detected with peroxidase-conjugated

B Comparison of amino acid sequence of variable region in NPs

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HTNV   KDWSDRIEQWLIEPCKLLPDTAAVSLLGGPATNRDYLRQRQVALGNMETKESKAIRQHAEAAGCSMIEDIESP
AMRV   KDWGDRIEQWLSEPCKLLPDTAAVSLHGGXATNRDYLRQRQAALGNMETKESKAIRQHAETAGCSMIEDIESP
DBSV   KDWTDRIESWLGEPCKLLPDTSAVSLLGGPATNRDYIRQRQVALGNMETKEAKAVRQHAESAGCSMIDNIDSP

DOBV   KNWTERVEEWLDLPCKLLSEPSPTSLTKGPSTNRDYLNQRQGALAKMETKEAQAVRKHAIDAGCNLIDHIDSP
SAAV   KNWTERVEEWLDLPCKLLSEPSPTSLTKGPSTNRDYLSQRQGALAKMETKEAQVVRKHAIDAGCNIIDHIDSP

SEOV   KDWTSRIEEWLGAPCKFMAESLIAGSLSGNPVNRDYIRQRQGALAGMEPKEFQALRQHSKDAGCTLVEHIESP
Gou3   KDWTSRIEDWLGAPCKFMAESPIAGSLSGNPVNRDYIRQRQGALAGMEPKEFQALRQHSKDAGCTMVEQIESP

THAIV  KDWTDRIQDWLGAPCKFLPDLSTQSLHASPPTNADYIAQRQGLLAGMETKESKGIRDHARDAGCTLVDHLDSP
Camb.  KDWTDRIQDWLGSPCKFLPDLSTQSLHASPPTNADYIAQRQGLLAGMETKESKGIRDHARDAGCTLVDHLDSP
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Fig. 2 Reactivities of rodent sera to truncated N antigens of HTNV, SEOV, and THAIV and comparison of the amino acid sequences of the variable region in NP. **a** Hantavirus-infected rat and mouse sera were subjected to serotyping ELISA. Sera from laboratory rats immunized with SEOV strain SR-11 or Gou3 were prepared previously [14], and serum from rats immunized with THAIV and non-immunized rat serum (NRS) were used for serotyping ELISA (*upper left*). Wild greater bandicoot rats A2-172 and A2-165 were both reported previously to have anti-THAIV antibody [7], and B2-079 serum was used as a negative control (*upper right*). The lower panel shows immune mouse sera to THAIV strain thai749, HTNV strain 76118 [14], DBSV strain NC167 [14], and SEOV strain SR-11 [14]. **b** Comparison of the amino acid sequences of the variable region of the NPs. The regions from aa 230–302 of representative Murinae-associated hantaviruses are shown for HTNV strain 76118 (M14626), AMRV strain AP61 (AB071183), DBSV strain NC167 (AB027523), DOBV strain Af19 derived from *Apodemus flavicollis* (AJ410615), Saaremaa virus SAAV strain 160 V derived from *Apodemus agrarius* (AJ009773), SEOV strains SR11 (M34881) and Gou3 (AB027522), THAIV strain thai749 (AB186420), and Cambodian hantavirus strain from *R. rattus* (AJ427511)

mouse anti-rat IgG (H + L) (Zymed, South San Francisco, CA, USA) and an *o*-phenylenediammonium dichloride (OPD) substrate tablet (Sigma, St. Louis. MO, USA). We also established an ELISA system for mouse sera. A 96-well plate was coated directly with recombinant and truncated NPs and negative control antigen, without capture antibody. The bound antibodies were detected with peroxidase-conjugated goat anti-mouse IgG (Zymed) and OPD substrate tablets (Sigma). The amounts of the antigens were adjusted based on the ELISA OD value against pooled non-immunized mouse sera.

As shown in Fig. 2a, immune rodent sera against THAIV showed a typical THAIV infection reactivity pattern, while anti-SEOV immune sera against strains SR-11 (derived from *Rattus norvegicus*) and Gou3 [14] (derived from *R. rattus*) showed a typical SEOV infection reactivity

pattern. Similarly, immune sera to Da Bie Shan virus (DBSV) strain NC167 also showed a typical HTNV infection reactivity pattern. Figure 2b shows a comparison of the aa sequence in the type-specific region of the NP, which is thought to contain type-specific epitopes (aa 230–302). In fact, two PUUV-specific MAbs (3H9 and 5F4) were reported to bind to this region [18, 19]. From the sequence comparison, we identified four subgroups among Murinae-associated hantaviruses: HTNV, SEOV, DOBV, and THAIV. The HTNV subgroup includes DBSV and Amur virus (AMRV), which was recognized in far eastern Russia as a pathogenic hantavirus [20], and its animal reservoir is *Apodemus peninsulae* [21]. AMRV and other *A. peninsulae*-borne hantavirus from Korea (Soochong virus [22]) and China (lineage #2 [14]) were identified as distinct lineage from classical *A. agrarius*-borne HTNV. Previously, we identified AMRV patient sera using HTNV serotyping antigen [23]. Similarly, strains Gou3 and SR11 were found within the SEOV subgroup. In this study, both human and rodent antisera to THAIV showed THAIV-specific reactions in serotyping ELISA. In contrast, the Cambodian strain from black rats (*R. rattus*) [24] had an aa sequence similar to that of THAIV in this type-specific region. These observations indicated that THAIV and its relatives occur in both the greater bandicoot rat and black rat in South East Asia. Therefore, the serotyping antigen for THAIV developed in this study is useful for detecting THAIV and relative viruses from Cambodia and differentiating them from other hantaviruses.

Recently, there have been gradual increases in the number of case reports of hantavirus infection in Asian countries, including Thailand [7], Indonesia [25], Vietnam [26], and India [27, 28]. In terms of public health, it is important to develop rapid, convenient methods for epidemiological surveillance and studies. Our system will become a valuable tool for surveying human and rodent cases of THAIV infection. However, the reliability of the system is uncertain because we tested only two serum samples from one patient. More serum samples from THAIV patients are needed to assess the applicability of our serotyping ELISA system.

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References

1. Nichol ST, Beaty BJ, Elliott RM, Goldbach R, Plyusnin A, Schmaliohn CS, Tesh RB (2005) Bunyaviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) Virus taxonomy: classification and nomenclature of viruses: eighth report of the international committee on the taxonomy of ciruses. Elsevier, San Diego, pp 695–716
2. Jackson AP, Charleston MA (2004) A cophylogenetic perspective of RNA-virus evolution. Mol Biol Evol 21:45–57
3. Schmaljohn C, Hjelle B (1997) Hantaviruses—a global disease problem. Emerg Infect Dis 3:95–104
4. Elwell MR, Ward GS, Tingpalapong M, LeDuc JW (1985) Serologic evidence of Hantaan-like virus in rodents and man in Thailand. Southeast Asian J Trop Med Public Health 16:349–354
5. Chu YK, Rossi C, Leduc JW, Lee HW, Schmaliohn CS, Dalrymple JM (1994) Serological relationships among viruses in the hantavirus genus, family Bunyaviridae. Virology 198:196–204
6. Xiao S-Y, Leduc JW, Chu YK, Schmaliohn CS (1994) Phylogenetic analyses of virus isolates in the genus *hantavirus*, Family *Bunyaviridae*. Virology 198:205–217
7. Pattamadilok S, Lee B-H, Kumperasart S, Yoshimatsu K, Okumura M, Nakamura I, Araki K, Khoprasert Y, Dangsupa P, Panlar P, Jandrig B, Kruger DH, Klempa B, Jakel T, Schmidt J, Ulrich R, Kariwa H, Arikawa J (2006) Geographical distribution of hantaviruses in Thailand and potential human health significance of Thailand virus. Am J Trop Med Hyg 75:994–1002
8. Suputthamongkol Y, Nitatpattana N, Chayakulkeeree M, Palabodeewat S, Yoksan S, Gonzalez JP (2005) Hantavirus infection in Thailand: first clinical case report. Southeast Asian J Trop Med Public Health 36:217–220
9. 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. J Clin Microbiol 39:2397–2404
10. Arikawa J, Takashima I, Hashimoto N (1985) Cell fusion by haemorrhagic fever with renal syndrome (HFRS) viruses and its application for titration of virus infectivity and neutralizing antibody. Arch Virol 86:303–313
11. 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
12. 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 77:695–704
13. Yoshimatsu K, Lee BH, Araki K, Morimatsu M, Ogino M, Ebihara H, Arikawa J (2003) The multimerization of hantavirus nucleocapsid protein depends on type-specific epitopes. J Virol 77:943–952
14. Wang H, Yoshimatsu K, Ebihara H, Ogino M, Araki K, Kariwa H, Wang Z, Luo Z, Li D, Hang C, Arikawa J (2000) Genetic diversity of hantaviruses isolated in china and characterization of novel hantaviruses isolated from *Niviventer confucianus* and *Rattus rattus*. Virology 278:332–345
15. 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
16. Okumura M, Yoshimatsu K, Araki K, Lee BH, 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. Arch Virol 149:2427–2434
17. Ogino M, Ebihara H, Lee B-H, Araki K, Lundkvist A, Kawaoka Y, Yoshimatsu K, Arikawa J (2003) Use of vesicular stomatitis virus pseudotypes bearing Hantaan or Seoul virus envelope proteins in a rapid and safe neutralization test. Clin Diagn Lab Immunol 10:154–160

18. Lundkvist A, Bjorsten S, Niklasson B, Ahlborg N (1995) Mapping of B-cell determinants in the nucleocapsid protein of Puumala virus: definition of epitopes specific for acute immunoglobulin G recognition in humans. Clin Diagn Lab Immunol 2:82–86
19. 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
20. Yashina LN, Patrushev NA, Ivanov LI, Slonova RA, Mishin VP, Kompanez GG, Zdanovskaya NI, Kuzina II, Safronov PF, Chizhikov VE, Schmaljohn C, Netesov SV (2000) Genetic diversity of hantaviruses associated with hemorrhagic fever with renal syndrome in the far east of Russia. Virus Res 70:31–44
21. Lokugamage K, Kariwa H, Hayasaka D, Cui BZ, Iwasaki T, Lokugamage N, Ivanov LI, Volkov VI, Demenev VA, Slonova R, Kompanets G, Kushnaryova T, Kurata T, Maeda K, Araki K, Mizutani T, Yoshimatsu K, Arikawa J, Takashima I (2002) Genetic characterization of hantaviruses transmitted by the Korean field mouse (*Apodemus peninsulae*), far east Russia. Emerg Infect Dis 8:768–776
22. Baek LJ, Kariwa H, Lokugamage K, Yoshimatsu K, Arikawa J, Takashima I, Kang JI, Moon SS, Chung SY, Kim EJ, Kang HJ, Song KJ, Klein TA, Yanagihara R, Song JW (2006) Soochong virus: an antigenically and genetically distinct hantavirus isolated from *Apodemus peninsulae* in Korea. J Med Virol 78:290–297
23. Miyamoto H, Kariwa H, Araki K, Lokugamage K, Hayasaka D, Cui BZ, Lokugamage N, Ivanov LI, Mizutani T, Iwasa MA, Yoshimatsu K, Arikawa J, Takashima I (2003) Serological analysis of hemorrhagic fever with renal syndrome (HFRS) patients in far eastern Russia and identification of the causative hantavirus genotype. Arch Virol 148:1543–1556
24. Reynes JM, Soares JL, Hue T, Bouloy M, Sun S, Kruy SL, Flye Sainte Marie F, Zeller H (2003) Evidence of the presence of Seoul virus in Cambodia. Microbes Infect 5:769–773
25. Plyusnina A, Ibrahim IN, Winoto I, Porter KR, Gotama IB, Lundkvist A, Vaheri A, Plyusnin A (2004) Identification of Seoul hantavirus in *Rattus norvegicus* in Indonesia. Scand J Infect Dis 36:356–359
26. Truong T-T, Truong U-N, Yoshimatsu K, Lee B-H, Araki K, Arikawa J (2004) Report of serology hantavirus in human and rodent at vietnam in 2003. In: The 6th international conference on hemorrhagic fever with renal syndrome (HFRS), hantavirus pulmonary syndrome (HPS) and hantaviruses. Seoul, p 146
27. Chandy S, Mitra S, Sathish N, Vijayakumar TS, Abraham OC, Jesudason MV, Abraham P, Yoshimatsu K, Arikawa J, Sridharan G (2005) A pilot study for serological evidence of hantavirus infection in human population in south India. Indian J Med Res 122:211–215
28. Clement J, Maes P, Muthusethupathi M, Nainan G, van Ranst M (2006) First evidence of fatal hantavirus nephropathy in India, mimicking leptospirosis. Nephrol Dial Transplant 21:826–827

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FULL PAPER

Genetic and antigenic analyses of a Puumala virus isolate as a potential vaccine strain

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Abstract

Puumala virus (PUUV), a causative agent of hemorrhagic fever with renal syndrome (HFRS), is prevalent in Europe and European Russia. No vaccine has been developed for PUUV-associated HFRS, primarily because of the low viral yield in cultured cells. A PUUV strain known as DTK/Ufa-97 was isolated in Russia and adapted for growth in Vero E6 cells maintained in serum-free medium. The DTK/Ufa-97 strain produced a higher viral titer in serum-free medium, suggesting that it may prove useful in the development of an HFRS vaccine. When PUUV-infected Vero E6 cells were grown in serum-free medium, the DTK/Ufa-97 strain yielded more copies of intracellular viral RNA and a higher viral titer in the culture fluid than did the Sotkamo strain. Phylogenetic analysis revealed that PUUVs can be classified into multiple lineages according to geographical origin, and that the DTK/Ufa-97 strain is a member of the Bashkiria-Saratov lineage. The deduced amino acid sequences of the small, medium, and large segments of the DTK/Ufa-97 strain were 99.2% to 100%, 99.3% to 99.8%, and 99.8% identical, respectively, to those of the Bashkirian PUUV strains and 96.9%, 92.6%, and 97.4% identical, respectively, to those of the Sotkamo strain, indicating that the PUUVs are genetically diverse. However, DTK/Ufa-97 and other strains of PUUV exhibited similar patterns of binding to a panel of monoclonal antibodies against Hantaan virus. In addition, diluted antisera (i.e., ranging from 1:160 to 1:640) specific to three strains of PUUV neutralized both homologous and heterologous viruses. These results suggest that the DTK/Ufa-97 strain is capable of extensive growth and is antigenically similar to genetically distant strains of PUUV.

Key words: hantavirus, hemorrhagic fever with renal syndrome, Puumala virus, vaccine

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Introduction

Hantaviruses belong to the genus *Hantavirus*, within the family *Bunyaviridae*. These viruses cause two zoonoses: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Hemorrhagic fever with renal syndrome is caused by Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), Dobrava-Belgrade virus (DOBV), and Amur virus (AMRV), and occurs primarily in Asia and Europe. HPS occurs in the Americas and is caused by Sin Nombre virus (SNV), Andes virus (ANDV) and other hantaviruses[14,24,26]. Hantaviruses are transmitted via aerosolized excretions of rodents in the family *Muridae*. Their viral genomes contain large (L), medium (M), and small (S) segments of negative-stranded RNA, which encode a viral RNA-dependent RNA polymerase, a glycoprotein precursor, and a nucleocapsid protein (NP), respectively[27].

Five viruses are known to cause human HFRS in Russia. Specifically, PUUV and DOBV cause HFRS in European Russia[31], while HTNV, SEOV, and AMRV cause HFRS in Far Eastern Russia[18,21,29,36]. Sporadic cases of PUUV-and DOBV-induced HFRS were recently detected in the western Siberian regions of Russia[35]. The principal hosts for HTNV, AMRV, SEOV, and PUUV are *Apodemus agrarius, Apodemus peninsulae, Rattus norvegicus,* and *Myodes glareolus,* respectively. Detailed phylogenetic analyses of strains from Europe have shown that DOBV strains derived from *Apodemus flavicollis* form a separate evolutionary lineage (i.e., DOBV-Af), while strains derived from *A. agrarius* are more diverse. Strains from central Europe and central European Russia form the DOBV-Aa lineage, and are distinct from the Saaremaa strains of northeastern Europe[10,11]. In the Sochi district of southern Russia, a previously unknown DOBV variant (i.e., DOBV-Ap) was identified in *Apodemus ponticus,* a novel hantavirus host, and determined to cause HFRS[12,32]. Although the DOBV strains from *Apodemus* hosts in European Russia and Europe share high amino acid sequence similarity, phylogenetic analyses in humans and an animal model reveal that they form separate lineages with distinct virulence traits[13]. A novel DOBV-Ap lineage associated with *A. ponticus* emerged in an area south of European Russia, confirming the reputation of DOBV as the most virulent of the European hantaviruses[12].

Approximately 200,000 cases of HFRS are reported worldwide each year[4], including 150,000 cases in China[3,16], 600 to 1,000 cases in Korea[8], 1,000 cases in Finland, and 200 cases in Sweden[22,33]. In Russia, HFRS has the highest incidence and morbidity of all human zoonotic virus infections. Approximately 6,000 to 8,000 clinical cases of HFRS are reported in European Russia every year. Of these, most are caused by PUUV and a smaller fraction are caused by DOBV. As the strains of HFRS in European Russia are caused by several distinct hantaviruses and vary in severity, it is inappropriate to refer to the disease as 'nephropathia epidemica'. Consequently, the WHO Working Group proposed the term 'hemorrhagic fever with renal syndrome' to describe similar clinical syndromes in Russia, Europe and Asia[34].

HFRS can be prevented by reducing exposure to live rodents and their excreta. However, rodent control measures are expensive and difficult to maintain over long periods, as it would be impossible to completely eradicate the viral hosts. Hence, immunization would be the most effective way to decrease HFRS morbidity in endemic regions of Russia.

Several commercial hantavirus vaccines are produced in China and Korea[4,37]. These vaccines are effective against HTNV and SEOV infections, but do not provide immunity to antigenically distinct PUUVs. No vaccine has been developed against PUUV, primarily because of the low viral yield in cultured cells. A potential vaccine strain (i.e., DTK/Ufa-97) was isolated from a patient with HFRS during a large 1997 outbreak in the Bashkiria region of Russia[23]. High titers of the virus were prepared in Vero E6 cells grown in serum-free medium (SFM). Here, we perform genetic and antigenic characterizations of the DTK/Ufa-97 strain.

Materials and Methods

Cell lines and culture media: Vero E6 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultivated in Eagle's minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine (Sigma, St. Louis, MO, USA), 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (penicillin-streptomycin) (Cambrex, East Rutherford, NJ, USA). Vero E6 cells were adapted to SFM via growth in virus production (VP)-SFM (Invitrogen) containing 2 mM L-glutamine for 2 months.

Viral strains: The potential vaccine strain (i.e., DTK/Ufa-97) was isolated in 1997 from a deceased HFRS patient in Bashkiria, Russia and was adapted to Vero E6 cells grown in SFM. Two other PUUV strains were used, including a prototype Sotkamo strain that originated from *M. glareolus* in Finland[28] and a Kazan strain that originated from *M. glareolus* in Kazan, Russia[6,19]. Strains 76-118[15], SR-11[9], and H5[18] were used as representative HTNV, SEOV, and AMRV strains, respectively. All virus strains were propagated in Vero E6 cells prior to use.

Plaque assay: Freshly trypsinized Vero E6 cells (i.e., 1×10^6 cells/well) were seeded into the flat-bottom wells of six-well Multiwell Cell Culture Plates (BD Biosciences, San Jose, CA, USA). The medium was aspirated from the cultures and 0.2 ml aliquots of serial 10-fold viral dilutions were inoculated into the wells. The viruses were allowed to adsorb for 1 hr at 37℃, whereupon 10 ml of an overlay mixture [i.e., Eagle's MEM "Nissui 1" (Nissui Pharmaceutical Co., Ltd., Ueno, Tokyo, Japan) supplemented with 2 mM L-glutamine (Sigma), 10% FBS, and 1.5% SeaKem GTG Agarose (Cambrex)] was added to each well. After one week, 2 ml of a 0.025% solution of Neutral Red (Wako, Osaka, Japan) in overlay medium was added to each well. The wells were examined for plaques 7 days after staining.

Sampling for analysis of PUUV replication: Vero E6 cells grown in MEM and Vero E6 cells adapted to SFM were used to assess PUUV replication. The cells were infected with DTK/Ufa-97 or Sotkamo and cultured for 21 days. Culture fluids and infected cells were collected at 2, 6, 12, and 21 hours post-infection and at 3, 7, 10, 14, 17, and 21 days post-infection (dpi). The culture medium was changed every 7 days. The collected fluids were centrifuged at 1,200 rpm for 5 min, and the supernatants were stored at -80℃ as viral stocks. Infected cells were collected in MEM or SFM using a cell scraper. The cells were then suspended and centrifuged at 1,200 rpm for 5 min. The resulting cell pellets were stored at -80℃ until further use.

Indirect immunofluorescence assay: Monoclonal antibodies (MAbs) specific to glycoproteins Gn and Gc of strain HTNV 76-118[1] were obtained from mouse ascitic fluid and used in indirect immunofluorescence assay (IFA) for the antigenic characterization of DTK/Ufa-97. Hantavirus-infected Vero E6 cells were spotted onto 24 well slides. The slides were incubated for 4 hr at 37℃, fixed in cold acetone for 20 min, washed in phosphate-buffered saline (PBS) and distilled water, air dried, and stored at -40℃ until further use. Diluted MAbs (i.e., 1:10 to 1:1,000,000) derived from the hybridoma or ascitic fluids were spotted onto the slides, which were then incubated for 1 hr at 37℃ and washed three times with PBS. The slides were then incubated in Alexa Fluor 488-conjugated goat anti-mouse IgG (i.e., final dilution =1:1,000; Invitrogen) for 1 hr at 37℃. The slides were washed and 90% glycerol was applied. The IFA titer of each MAb was expressed as the reciprocal of the maximum antibody dilution that yielded granular and scattered fluorescence in the cytoplasm.

Focus assay and titration of viruses: Approximately 2×10^5 Vero E6 cells/ml MEM (i.e., 0.5 ml/well) were seeded into eight chamber slides (Iwaki, Nihonbashi, Tokyo, Japan), maintained in a CO_2 incubator overnight, and infected with serially di-

luted stocks of Sotkamo or DTK/Ufa-97 strains. After adsorption for 1 hr in a CO_2 incubator, the virus inoculum was removed and MEM containing 1.5% carboxymethyl cellulose sodium salts (Wako) was layered onto the cells at a concentration of 0.6 ml/well. The cells were then cultured in a CO_2 incubator for 14 days at 37℃.

The resulting viral foci were visualized by IFA. Briefly, the cultured Vero E6 cells were washed three times with PBS, fixed with 0.2 ml/well of methanol for 20 min under UV light in a safety cabinet in a BSL3 laboratory. After removing methanol, the slides were thoroughly air-dried, and washed with PBS. The slides were then incubated in anti-PUUV hamster serum (i.e., final dilution=1:1,000) for 1 hr at 37℃, washed, and incubated with Alexa Fluor 488-conjugated goat anti-hamster IgG (i.e., final dilution=1:1,000; Invitrogen) for 1 hr. After washing, 90% glycerol was applied to the slides, and the viral foci were counted and measured under a fluorescence microscope. The focus diameters were expressed in μm, and the viral titers were expressed as focus-forming units/ml (i.e., ffu/ml).

Focus reduction neutralization test: The endpoint titers of the neutralizing antibodies were determined using focus reduction neutralization test (FRNT). Hamster immune sera specific to PUUV strains DTK/Ufa-97, Kazan, and Sotkamo; and mouse immune sera specific to HTNV strain 76-118, AMRV strain H5, and SEOV strain SR-11 were used to compare the antigenicity of DTK/Ufa-97 to that of other PUUV and hantavirus strains. Serial 2-fold dilutions of immune sera (30 μl) were mixed with equal volumes of viral stock (i.e., 60 ffu /30 μl) and incubated for 1 hr at 37℃. The mixture was then used to inoculate Vero E6 cell monolayers grown in 96-well flat-bottom plates at a concentration of 50 μl/well (Nunc TM, Roskilde, Denmark). After adsorption for 1 hr at 37℃, the inocula were removed and MEM containing 1.5% carboxymethyl cellulose sodium salts was layered onto the cells (i.e., 200 μl/well). The cells were cultured in a CO_2 incubator for 7 days at 37℃, washed with PBS, fixed with methanol, and air-dried.

The fixed cells were incubated with MAb E5/G6 (i.e., final dilution=1:200)[38] for 1 hr at 37℃. After three washes with PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (i.e., final dilution=1:1,000) for 1 hr at 37℃. The stained foci were counted under a fluorescence microscope, and the FRNT titer was defined as the highest dilution of serum associated with at least an 80% reduction in focus formation.

RNA isolation and reverse transcription: Total RNA was isolated from DTK/Ufa-97-infected Vero E6 cells using Isogen (Nippon Gene), according to the manufacturer's protocol. In preparation for first-strand cDNA synthesis, 11 μl of the extracted RNA (i.e., 5 μg) was mixed with 1 μl of random primers (i.e., 3 μg/μl, Invitrogen) and 1 μl of 10 mM dNTPs (TaKaRa, Otsu, Japan). The mixture was heated at 70℃ for 10 min, cooled to 25℃ over the span of 10 min, and chilled on ice for 3 min. Reverse transcription was performed via the addition of 4 μl of 5× first-strand buffer (Invitrogen), 2 μl of 0.1 mM DTT, and 1 μl of SuperScript II (200 U/μl, Invitrogen). The cDNA synthesis reaction was allowed to proceed for 50 min at 42℃, and was stopped by heating at 70℃ for 15 min.

Real-time polymerase chain reaction: Before cDNA synthesis for real-time polymerase chain reaction (PCR), RNA was treated with DNase. Briefly, 15 μg RNA was mixed with 5 μl of 10× DNase buffer (TaKaRa), 2 μl of RNase-free DNase I (5 U/μl; TaKaRa), 0.5 μl of RNase Out ribonuclease inhibitor (40 U/μl; Invitrogen), and double-distilled water (DDW) to a final volume of 50 μl. The tubes were incubated for 30 minutes at 37℃, precipitated with lithium chloride (Ambion, Austin, TX, USA), and dissolved in 30 μl of DDW. The DNase-treated RNA was used for cDNA synthesis as described above.

Real-time PCR was then performed on the DNase-treated samples. Primers and minor groove binder (MGB) probes specific to the PUUV

S segment were designed using Primer Express software (ver. 2.0; Applied Biosystems, Foster City, CA, U.S.A.), and probes were labeled with 5' reporter dye, 6-Carboxyfluorescein (FAM) and a 3'-MGB/non-fluorescent quencher. After optimization of the primer and probe concentrations, samples were assayed in quadruplicate 25 μl reactions. Each reaction contained 2.25 μl of cDNA, 12.5 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.225 μl each of 100 μM forward and reverse primers (i.e., Sotkamo62Fw: 5' -TCCAAGA GGATATAACCCGCCAT-3' and Sotkamo257Rv: 5'-TTCCTGGACACAGCATCTGC-3', respectively), 0.46 μl of 10.9 μM fluorescent probe (i.e., Sotkamo 194: 5'-TGTCAGCACTGGAGGA-3'), and 9.34 μl of DDW. Samples were incubated at 50℃ for 2 min and 95℃ for 10 min, followed by 60 thermal cycles of 95℃ for 15 sec and 60℃ for 1 min. Real-time data were collected using the 7000 Sequence Detection System (Applied Biosystems).

Real-time PCR data were normalized to rodent GAPDH expression. The same amount of cDNA (i.e. 2.25 μl) was mixed with 12.5 μl of 2× TaqMan Universal PCR Master Mix, 0.25 μl each of 10 μM rodent GAPDH forward and reverse primers, and 0.25 μl of 20 μM rodent GAPDH probe (VIC-labeled). All primers and probes were purchased from Applied Biosystems.

Nucleotide sequencing analysis: The cDNA derived from the total RNA of DTK/Ufa-97-infected Vero E6 cells was amplified using Platinum® Taq DNA polymerase high fidelity (Invitrogen), according to the manufacturer's instructions. The reaction mixture also contained 2 pmol of primers specific to the S, M, and L segments of the DTK/Ufa-97 strain, in a final volume of 25 μl. After an initial denaturation step (i.e., 94℃ for 2 min), the cDNA was amplified via 35 thermal cycles of 94℃ for 30 sec, 55℃ for 30 sec, and 68℃ for 4 min.

The 3'- and 5'-ends of the S, M, and L segments were amplified using RNA isolated from the infected-cell culture medium and the 5' RACE System for Rapid Amplification of cDNA Ends (ver. 2.0; Invitrogen). In preparation for 5'-end amplification, randomly primed synthetic cDNA was dCTP-tailed using a terminal deoxynucleotidyl transferase. The tailed cDNA was amplified using a 5' RACE abridged anchor primer (AAP, Invitrogen) and PUUV-specific primers. In preparation for 3'-end amplification, the isolated RNA was CTP -tailed using poly(A) polymerase (Ambion), and the tailed RNA was reverse-transcribed using AAP and SuperScript™ II (Invitrogen). The cDNA was amplified using PUUV-specific primers and an abridged universal amplification primer (Invitrogen).

The amplified products were electrophoresed in agarose gels, stained with ethidium bromide, and visualized under UV light. The DNA fragments were excised from the gel and purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA), according to the manufacturer's instructions. Purified DNA fragments were directly sequenced at least two times in the forward and reverse directions using the ABI-PRISM Dye Terminator Sequencing Kit and the ABI 3130 Genetic Analyzer (both from Applied Biosystems).

Phylogenetic analysis: Hantavirus nucleotide (nt) and deduced amino acid sequences were compared using Genetyx software (ver. 8). The ClustalX program package (ver. 2.0) was used to generate a phylogenetic tree using the neighbor-joining method with 1,000 bootstrap replicates.

Statistical analysis: The viral RNA copies in infected Vero E6 cells and virus titers in cultured media were compared by Student's t-test. P values of 0.05 or less were considered statistically significant.

Results

Plaque and focus formation by PUUV strain DTK/Ufa-97

To examine the plaque-and focus-forming abilities of DTK/Ufa-97, Vero E6 monolayers were inoculated with virus and the resulting plaques

and foci were enumerated. Vero E6 cells infected with DTK/Ufa-97 exhibited small plaques (average diameter=1 mm to 2 mm) at 14 days post-infection, whereas cells infected with the PUUV Sotkamo strain did not form plaques (data not shown). In addition, the DTK/Ufa-97 foci (average diameter ± S.D., 196 μm±46 μm) were more than twice as large as the Sotkamo foci (average diameter ± S.D., 80 μm±6 μm) (data not shown).

Viral RNA copy number and virus titer

The DTK/Ufa-97-infected Vero E6 cells and the corresponding culture media were collected at various intervals after infection, and the viral RNA copy number and viral titer were determined. Viral RNA expression remained unchanged in Vero E6 cells grown in MEM, with approximately 1×10^3 to 7×10^3 copies of viral RNA transcribed during the 24 hr following infection. The copy number increased to approximately 5×10^4 copies at 3 dpi and remained at that level until 21 dpi (Fig. 1). In comparison, Sotkamo RNA replication occurred more slowly, reaching a plateau level at 7 dpi. When SFM was used instead of MEM, the DTK/Ufa-97 RNA copy number increased to 2.4×10^5 by 14 dpi and remained at that level until 21 dpi. However, fewer than 4×10^3 copies of Sotkamo RNA were present at 21 dpi.

When Vero E6 cells were grown in MEM, the DTK/Ufa-97 viral titer reached a peak of 2.8×10^5 ffu/ml at 7 dpi, then decreased to 1.4×10^4 ffu/ml at 10 dpi and remained unchanged until 21 dpi (Fig. 2). In contrast, the Sotkamo titer reached a peak of 2.1×10^4 ffu/ml at 7 dpi, then declined to 6.0×10^3 ffu/ml at 10 dpi and remained unchanged until 21 dpi. When Vero E6 cells were grown in SFM, the DTK/Ufa-97 titer reached peaks of 4.5×10^4, 8.3×10^4, and 9.3×10^4 ffu/ml at 7, 14, and 21 dpi,

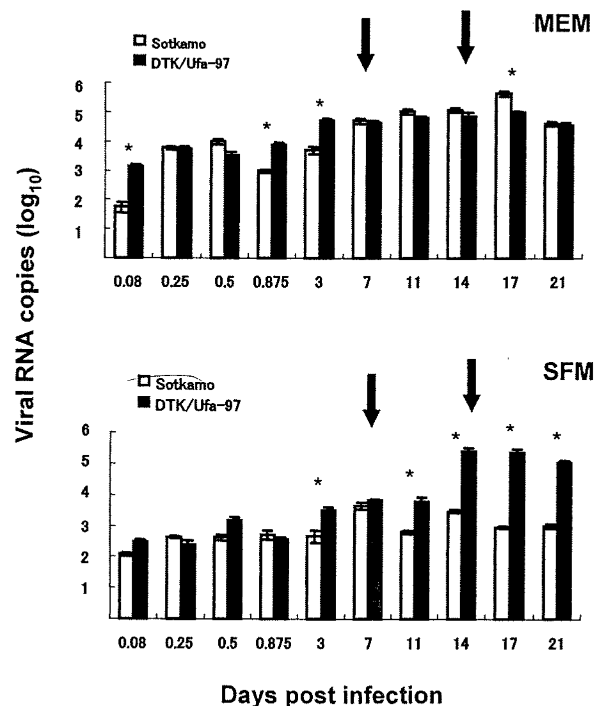

Fig. 1. Copy numbers of PUUV RNA in infected Vero E6 cells.
Cells were infected with PUUV strains Sotkamo and Ufa-97 at a multiplicity of infection (MOI) of 0.02, and were then maintained in MEM containing 10% FBS (*MEM*) or in *SFM*. Viral RNA copy numbers were determined via quadruplicate real-time PCR experiments. The number of viral RNA copies in infected cells is expressed as log10 of the RNA copy number. The standard deviation for each virus is shown at the top of the column corresponding to RNA copy number. Arrows indicate times when the medium was changed. Asterisks indicate significant differences, as determined using Student's t-tests (defined by $P < 0.05$).

respectively, whereas the Sotkamo titer remained less than 3.1×10^3 ffu/ml throughout the observation period.

Sequencing of the complete DTK/Ufa-97 genome

The full-length S, M, and L segments of the DTK/Ufa-97 genome were sequenced, and the resulting data were deposited into the DNA Data Bank of Japan under accession numbers AB 297665, AB297666, and AB297667, respectively. To our knowledge, this is the first complete genomic sequence of a human PUUV isolate from Russia. The S, M, and L segments of the DTK/Ufa-97 strain were 1,829, 3,682, and 6,550 nts in length, respectively (data not shown). These sequences differ from those of the Sotkamo genome only in the length of the S segment, which was 1 nt longer in Sotkamo strain (data not shown).

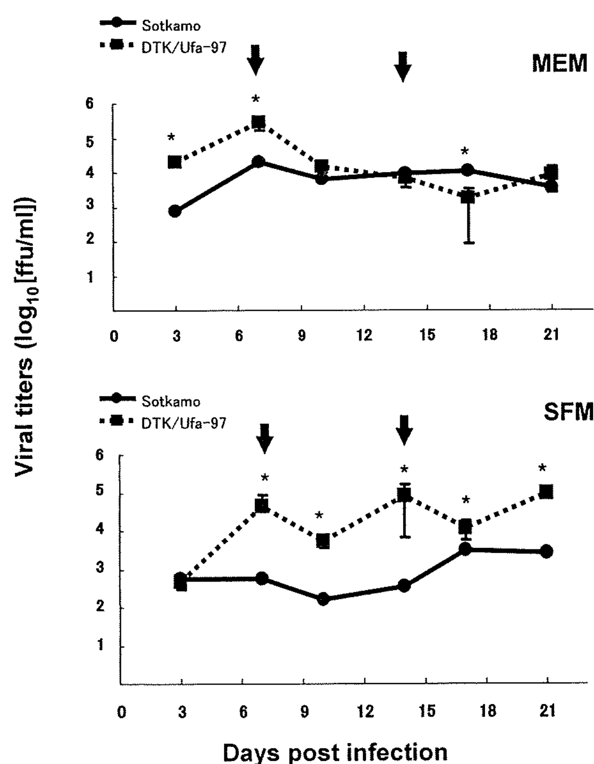

Fig. 2. Growth of PUUV strains.
Vero E6 cells were infected with Sotkamo or DTK/Ufa-97 at an MOI of 0.02 and maintained in either MEM or SFM. Supernatants were collected at the indicated times. The viral titer in the supernatant fraction was expressed as $\log_{10}$ [ffu/ml], and titers for each virus were determined in IFA triplicates. The standard deviation for each virus is shown at the top of the viral titer column. Arrows indicate the time points at which the medium was changed. Asterisks indicate significant differences, as determined using Student's t-tests (defined by $P < 0.05$).

Amino acid and nucleotide sequence comparisons

The nucleotide sequence of the DTK/Ufa-97 S segment was 99.9%, 100%, 99.5%, 99.5%, 93.9%, 93.9%, and 85.3% identical to the sequences of PUUV strains CG1820, P360, K27, CG17, Fs808, Kazan, and Sotkamo, respectively. Nucleotide sequence identity was greater than 93% among the Russian PUUVs; however, the Russian and Finnish PUUV Sotkamo strains were approximately 84% identical (Table 1). The predicted amino acid sequences of all of the PUUVs, including the Sotkamo strain, were more than 96% identical (Table 1).

The nucleotide sequence of the DTK/Ufa-97 M segment was 99.6%, 99.8%, 99.8%, 99.4%, 85.8%, 82.8%, and 80.3% identical to those of PUUV strains CG1820, P360, K27, CG17, Kazan, Sotkamo, and Umea/hu, respectively (Table 2). The M segments of the Bashkirian viruses, including DTK/Ufa-97, were more than 99% identical at the nucleotide level, whereas the M segments of the Bashkirian and Northern European strains were approximately 80.3% to 83.2% identical. The Russian PUUV M segments were more than 94.5% identical at the amino acid level, whereas the entire group of PUUV M segments were more than 88% identical (Table 2).

The PUUV L segments were more than 81% identical at the nucleotide level, while the amino acid sequences of the L segment were at least 93% identical among all PUUVs (Table 3).

Phylogenetic analyses

We examined the evolutionary relationship between DTK/Ufa-97 and other hantaviruses by performing phylogenetic analyses of the S, M, and L genome segments using the neighbor-joining method. The DTK/Ufa-97 strain was determined to be a member of the PUUV group, and all three

segments of the DTK/Ufa-97 strain were determined to be members of the Russia/Bashkiria-Saratov lineage (Figs. 3A and 3B). The PUUV branching patterns were consistent with geographical origin, and PUUVs from European Russia (i.e., including the Bashkiria-Saratov, Tataria, and Samara PUUVs from the Volga river region) occupied a single cluster. The Russian/Omsk PUUVs clustered with the Sotkamo strain (Fig. 3A).

Table 1. Nucleotide and amino acid identities of the hantavirus S segment[a].

		ucleotide identities (%)									
		Puumala virus (PUUV)								HTNV[b]	SNV[c]
		Ufa-97[d,e]	CG1820	P360[e]	K27[e]	CG17	Fs808[e]	Kazan	Sot[f]	76-118	NM-H10
VUUP	Ufa-97	–	99.9	100	99.5	99.5	93.9	93.9	85.3	62.2	67.8
VUUP	CG1820	99.7	–	99.9	99.4	94.4	93.8	93.8	85.2	62.1	67.8
VUUP	P360	100	99.7	–	99.5	99.5	93.9	93.9	85.2	62.2	67.7
VUUP	K27	99.2	98.9	99.2	–	99.1	93.5	93.5	85.0	62.0	67.3
VUUP	CG17	100	99.7	100	99.2	–	94.2	94.4	85.5	62.7	67.5
VUUP	Fs808	99.4	99.2	99.4	98.6	99.4	–	94.8	84.6	62.6	67.8
VUUP	Kazan	98.9	98.6	98.9	98.0	98.9	98.9	–	84.5	62.5	68.1
VUUP	Sotkamo	96.9	96.6	96.9	96.3	96.9	96.9	96.8	–	63.3	66.7
	HTNV	59.3	59.0	59.3	58.4	59.3	59.6	59.3	59.6	–	61.3
	SNV	69.7	69.9	69.7	69.1	69.7	69.9	69.9	69.1	61.4	–
		Amino acid identities (%)									

[a] Nucleotide region to be compared : nt 172-1,239
[b] Hantaan virus
[c] Sin Nombre virus
[d] DTK/Ufa-97 strain
[e] PUUV originated from HFRS patients
[f] Sotkamo strain

Table 2. Nucleotide and amino acid identities of the hantavirus M segment[a].

		Nucleotide identities (%)									
		Puumala virus (PUUV)								HTNV[b]	SNV[c]
		Ufa-97[d,e]	CG1820	P360[e]	K27[e]	CG17	Kazan	Sot[f]	Umea[e]	76-118	NM-H10
VUUP	Ufa-97	–	99.6	99.8	99.8	99.4	85.8	82.8	80.3	60.0	66.7
VUUP	CG1820	99.3	–	99.5	99.5	99.1	85.7	82.8	80.3	60.1	66.7
VUUP	P360	99.8	92.3	–	99.9	99.3	86.0	83.1	80.5	60.2	66.9
VUUP	K27	99.7	99.1	99.7	–	99.4	86.3	83.2	80.8	60.5	67.3
VUUP	CG17	99.6	99.0	99.6	99.6	–	86.0	83.1	80.5	60.1	66.6
VUUP	Kazan	95.0	94.5	95.1	96.8	95.2	–	84.7	80.6	59.5	66.0
VUUP	Sotkamo	92.6	92.2	92.7	94.2	92.7	93.2	–	80.7	59.0	66.7
VUUP	Umea	88.9	88.4	88.9	91.1	89.1	89.4	89.3	–	59.8	65.9
	HTNV	53.4	51.9	52.2	54.0	53.3	54.5	53.6	51.8	–	58.9
	SNV	65.5	64.5	64.8	67.2	64.8	64.0	64.7	64.0	54	–
		Amino acid identities (%)									

[a] Nucleotide region to be compared: nt 53-3,494
[b] Hantaan virus
[c] Sin Nombre virus
[d] DTK/Ufa-97 strain
[e] PUUV originated from HFRS patients
[f] Sotkamo strain

Table 3. Nucleotide and amino acid identities of the hantavirus L segment[a].

		Nucleotide identities (%)							
		Puumala virus (PUUV)					HTNV[b]	SEOV[c]	SNV[d]
		Ufa-97[e,f]	CG1820	Kazan	Sot[g]	Umea[f]	76-118	80-39	NM-H10
PUUV	Ufa-97	–	99.8	87.1	84.5	81.3	67.0	67.1	71.0
PUUV	CG1820	99.8	–	87.1	84.6	81.4	67.0	67.2	71.0
PUUV	Kazan	99.1	98.9	–	85.0	81.9	67.1	66.9	71.7
PUUV	Sotkamo	97.4	97.2	97.4	–	82.1	66.8	67.8	71.3
PUUV	Umea	93.9	93.8	94.1	93.2	–	66.5	66.3	69.9
	HTNV	68.8	68.8	68.8	68.6	67.2	–	74.3	66.7
	SEOV	68.5	68.3	68.2	68.4	66.8	84.7	–	67.1
	SNV	77.8	77.1	77.4	77.6	76.1	68.9	68.9	–
		Amino acid identities (%)							

[a] Nucleotide region to be compared: nt 52-6,524
[b] Hantaan virus
[c] Seoul virus
[d] Sin Nombre virus
[e] DTK/Ufa-97 strain
[f] PUUV originated from HFRS patients
[g] Sotkamo strain

Table 4. Antigenic characteristic of Ufa-97 and other hantavirus strains.

MAbs against glycoprotein of HTNV	Antigenic site	IFA titer[a]					
		Puumala			Hantaan	Amur	Seoul
		Ufa-97[b]	Kazan	Sotkamo	76-118	H5	SR-11
8B6	Gn-a (1)	–	–	–	+++	+++	+
6D4	Gn-a (2)	–	–	–	–	–	–
10F11	Gn-a (2)	+	+	–	++	+++	+
2D5	Gn-b	–	–	–	++	++	+
3D5	Gn-b	–	–	–	+	+	+
16D2	Gn-b	–	–	–	++	++	–
HCO2	Gc-a (1)	–	–	–	++	++++	++++
16E6	Gc-a (2)	–	–	–	++	+++	++
EB06	Gc-b	+	+	+	++	+++	+
11E10	Gc-c	+++	+++	++	+++	+++	–
17G6	Gc-d	+	+	+	++	+++	+
5B7	Gc-d	++	+++	++	+++	+++	+++
20D3	Gc–e	+	+	–	++	+++	–
8E10	Gc-f (1)	+++	+++	+++	++	+++	++
1G8	Gc-f (1)	+++	+++	++	+++	+++	++
3B6	Gc-f (1)	+++	+++	+	+++	++	++
23G10-1	Gc-f (2)	–	–	–	++	++	++
7G6	Gc-f (2)	–	–	–	++	+++	++
18F5	Gc-f (2)	–	–	–	++	+++	+

[a] Antibody reactivity is defined as: –,<1:10; +,1:10; ++,1:100; +++,1:1,000; ++++,>1:1,000
[b] DTK/Ufa-97 strain

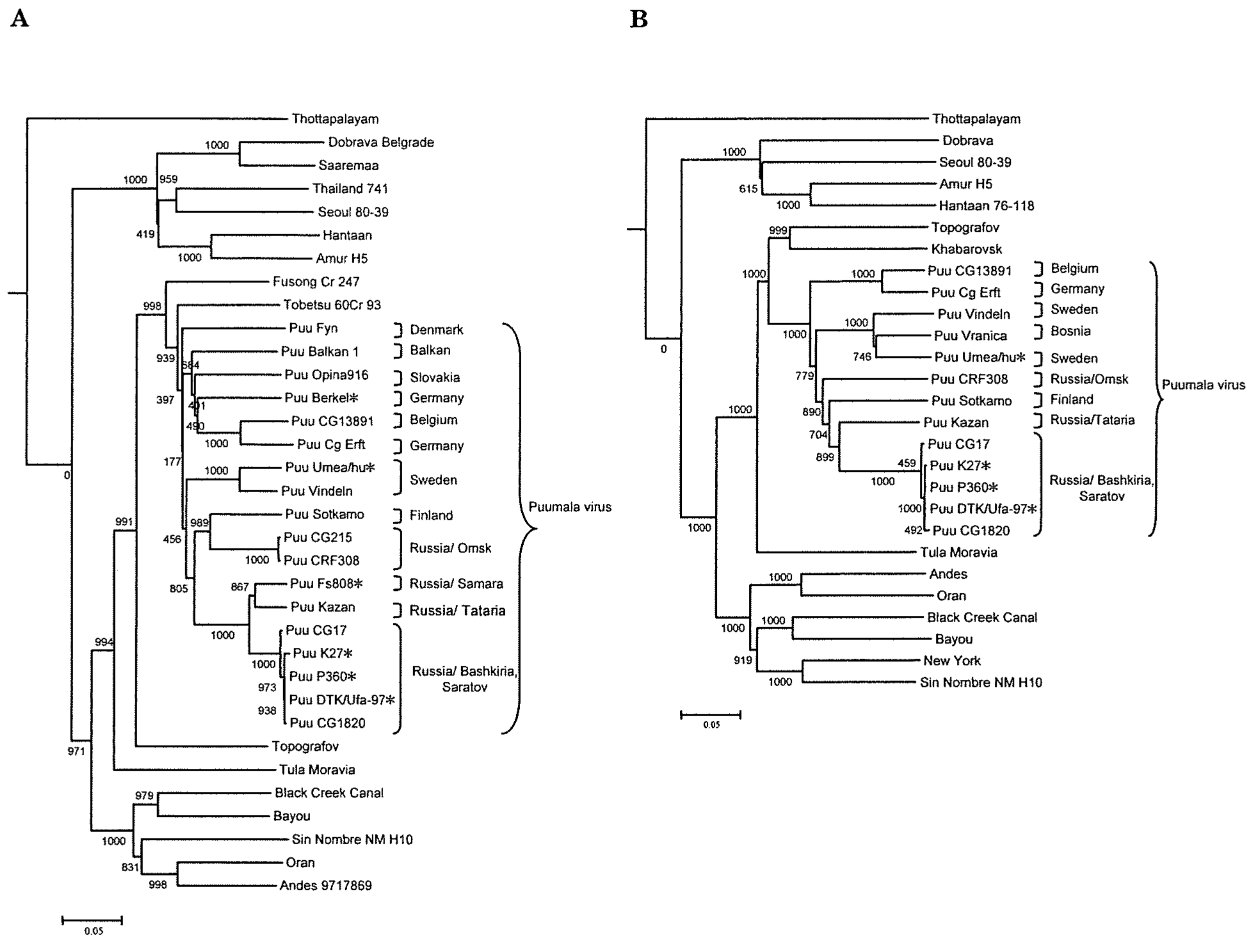

Fig. 3. Phylogenetic analysis of hantaviruses.
The nt sequences of the S, M, and L segments were obtained from the Genome Sequence Database. Multiple sequence alignment was performed using ClustalX software (ver. 2.0), and a phylogenetic tree was derived using the neighbor-joining method. Bootstrap values resulting from 1,000 replications are listed above each branch. A) Phylogenetic analysis of the 1068 nt S segment (i.e., approximately nucleotides 172 to 1239). The accession numbers for the S segment sequences are as follows: Thottapalayam, AY526097; Dobrava Belgrade, L41916; Saaremaa, AJ616854; Thailand 741, AB186420; Seoul 80-39, AY273791; Hantaan, M14626; Amur H5,AB127996; Fusong Cr 247, EF442087; Tobetsu 60Cr 93, AB010731; Puu Fyn, AJ238791; Puu Balkan1,AJ314600; Puu Opina 916, AF294652; Puu Berkel, L36943; Puu CG13891, U22423; Puu Cg-Erft, AJ238779; Puu Umea/hu, AY526219; Puu Vindeln/L20Cg/83, Z48586; Puu Sotkamo, X61035; Puu CG215, AF367066; Puu CRF308, AF367070; Puu F-s 808, AF411446; Puu Kazan, Z84204; Puu CG17/Baskiria-2001, AF442613; Puu K27, L08804; Puu P360, L11347; Puu DTK/Ufa-97, AB297665; Puu CG1820, M32750; Topografov, AJ011646; Tula Moravia/5302v/95, Z69991; Black Creek Canal, L39949; Bayou, L36929; Sin Nombre NM H10, L25784; Oran 22996, AF482715; and Andes Chile-9717869, AF291702. B) Phylogenetic tree of the 3442 nt viral M segment (i.e., approximately nucleotides 53 to 3494). The M segment accession numbers are as follows: Thottapalayam VRC-66412, EU001329; Dobrava-Belgrade DOBV/Ano-Poroia/Af19/1999, AJ410616; Seoul 80-39, S47716; Amur H5,AB127993; Hantaan 76-118, Y 00386; Topografov, AJ011647; Khabarovsk, AJ011648; Puu CG13891, U22418; Puu Cg-Erft, AJ238778; Puu Vindeln, Z49214; Puu Vranica, U14136; Puu Umea/hu, AY526218; Puu CRF308/Omsk, AF442617; Puu Sotkamo, X 61034; Puu Kazan, Z84205; Puu CG17/Baskiria-2001, AF442614; Puu K27, L08754; Puu P360, L08755; Puu DTK /Ufa97, AB297666; Puu CG1820, M29979; Tula Moravia/5302v/95, Z69993; Andes Chile-9717869, AF291703; Oran O122996, AF028024; Black Creek Canal, L39950; Bayou, L36930; New York NY-2,U36803; and Sin Nombre NM H10, L25783. Asterisks indicate viral strains of human origin.

Table 5. Cross-focus reduction neutralization titers of immune sera specific Ufa-97 and other representative hantaviruses.

Serum	Virus					
	Puumala			Hantaan	Amur	Seoul
	Ufa-97[a]	Kazan	Sotkamo	76-118	H5	SR-11
Ufa-97	160[b]	160	160	40	40	20
Kazan	160	160	320	80	80	20
Sotkamo	160	160	640	80	20	20
Hantaan	40	20	20	160	20	40
Amur	40	40	20	80	160	20
Seoul	20	<10	20	20	20	40

[a] DTK/Ufa-97 strain
[b] Neutralizing antibody titers to the homologous viruses are shaded and bold. Titers are shown as the reciprocal of the dilution that resulted in at least 80% reduction in focus, compared with the control (i.e., no antibody)

Antigenic characteristics of DTK/Ufa-97

The antigenic profile of the DTK/Ufa-97 strain was compared with that of other hantaviruses using IFA with a MAb panel. Six of the MAbs used in this study were specific to the HTNV envelope Gn glycoprotein, while 13 were specific to the Gc glycoprotein (Table 4). All MAbs specific to HTNV glycoproteins cross-reacted with AMRV, yielding very similar patterns. In addition, some MAbs specific to HTNV glycoproteins cross-reacted with SEOV, yielding somewhat dissimilar patterns (Table 4). Of the MAbs specific to antigenic sites of the Gn-a, Gn-b, Gn-a, and Gn-f(2) envelope glycoproteins, only MAb 10F11 cross-reacted with any of the PUUV strains. However, MAbs specific to the antigenic sites of the Gc-b, Gc-c, Gc-d, Gc-e, and Gc-f(1) glycoproteins reacted with all hantaviruses tested, including the PUUV strains (Table 4). In general, the PUUV strains exhibited similar reaction patterns to the latter group of MAbs (Table 4).

Cross-neutralization test

To further investigate the antigenic characteristics of DTK/Ufa-97, hamsters were infected with the DTK/Ufa-97, Kazan, and Sotkamo strains and immune sera were collected. These sera were then used to neutralize homologous or heterologous PUUV strains. All sera proved effective at high neutralizing titers (i.e., dilutions of 1:160 to 1:640) (Table 5). The PUUV immune sera exhibited lower neutralizing titers against HTNV, AMRV, and SEOV. However, immune sera specific to HTNV, AMRV, and SEOV exhibited high neutralizing antibody titers to homologous viruses but lower titers to heterologous viruses. These results indicate that the antigenic properties of DTK/Ufa-97 are similar to those of other PUUVs, with regards to the induction of neutralizing antibodies.

Discussion

Hemorrhagic fever with renal syndrome has the highest incidence and morbidity of all human zoonotic viral infections in Russia. Approximately 97% of HFRS cases are caused by PUUV in the European regions of Russia, whereas 3% of HFRS cases are caused by HTNV, SEOV and AMRV in the far-Eastern regions of the country[18,30]. An HFRS vaccine is urgently needed, as morbidity rates are high and approximately 12.5 million people (i.e., 25% of the population) in European regions are at risk for PUUV infection. Several hantavirus vaccines have been produced; however, none are effective against PUUV-induced HFRS. The difficulties in developing a PUUV vaccine stem from the low viral yield in cell culture. High titers of a potential vaccine strain, known as DTK/Ufa-97, can be cultured in SFM-grown Vero E6 cells. Here, we demonstrate that the DTK/Ufa-97 strain yields more viral RNA in infected Vero E6

cells and higher viral titers in the culture fluid of infected cells, when compared with the Sotkamo strain (Figs. 1 and 2). Our data clearly indicate that the DTK/Ufa-97 strain replicates more efficiently than the Sotkamo strain in SFM-cultured Vero E6 cells, as well as in MEM supplemented with FBS.

Minimum essential medium supplemented with FBS has been empirically shown to provide good conditions for cell growth and is commonly used in the formulation of growth media. However, FBS is a potential carrier of infectious agents such as fungi, bacteria, viruses, and prions, which could contaminate a final vaccine preparation[2]. Therefore, SFM is a safer alternative, as it does not contain components of animal or human origin. Several cell lines, including BHK-21[7] and Vero cells[17], have been successfully established in SFM.

A lot of PUUV sequences have been deposited in the DNA database, however, few full-length PUUV genome sequences are available, particularly for human isolates. Therefore, we determined the full-length nucleotide sequence of the DTK/Ufa-97 strain. To our knowledge, this is the first characterization of a Russian PUUV isolate from an HFRS patient. Our phylogenetic analysis revealed that the DTK/Ufa-97 strain is closely related to PUUV strains from the same geographic region (i.e., Bashkiria-Saratov) (Fig. 3). Our analysis identified four Russian PUUV clusters: Bashkiria-Saratov, Tataria, Samara, and Omsk (Fig. 3A). The first three clusters appeared on the same branch, while the Omsk cluster was more closely related to the Finland virus than to the other Russian viruses. This result is consistent with a previous report, in which the genetic identities of Russian and Finnish PUUV strains strongly correlated with their geographic origins[5,25].

The identities of predicted amino acid sequences in the S, M, and L segments were approximately 96.3%, 92.2%, and 93.2%, respectively among all PUUVs. However, the nucleotide sequences identities of the S, M, and L segments were much lower (i.e., approximately 84.5%, 80.3%, and 81.3%, respectively) (Tables 1-3). Few studies have examined the antigenic properties of PUUV; thus, we also examined the antigenic characteristics of DTK/Ufa-97 using MAbs. We compared the antigenicity of three PUUV strains (i.e., DTK/Ufa-97, Kazan, and Sotkamo), as well as other hantaviruses. The reaction patterns exhibited by the DTK/Ufa-97 strain were strikingly similar to those of other Russian and Finnish PUUVs (Table 4), but different than those of other hantaviruses, especially with regards to the Gn protein.

Envelope glycoproteins are presumed to play a major role in the induction of protective immunity and neutralizing antibodies[1,20]. Our cross-neutralization test demonstrated that a neutralizing antibody specific to the DTK/Ufa-97 strain also neutralized other PUUV strains at almost same antibody titer. In addition, antibodies to other PUUV strains cross-neutralized the DTK/Ufa-97 strain and homologous viruses (Table 5). Although the various PUUVs exhibit geographic-dependent genetic variation, they seem to share similar antigenic properties. Therefore, the DTK/Ufa-97 strain may prove useful in inducing protective immunity against a variety of PUUV strains, and may aid in the development of a DTK/Ufa-97-based vaccine.

Our findings revealed that the PUUV strain DTK/Ufa-97 grows well in Vero E6 cells cultured in SFM, and that it is antigenically similar to other PUUVs. These data may aid in the development of a PUUV vaccine strain based on DTK/Ufa-97.

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References

1) Arikawa, J., Schmaljohn, A. L., Dalrymple, J. M., and Schmaljohn, C. S. 1989. Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. *J. Gen. Virol.,* **70**: 615-624.
2) Butler, M., Burgener, A., Patrick, M., Berry, M., Moffatt, D., Huzel, N., Barnabe, N., and Coombs, K. 2000. Application of a serum-free medium for the growth of Vero cells and the production of reovirus. *Biotechnol. Prog.,* **16**: 854-858.
3) Chen, H. X., Qiu, F. X., Dong, B. J., Ji, S. Z., Li, Y. T., Wang, Y., Wang, H. M., Zuo, G. F., Tao, X. X., and Gao, S. Y. 1986. Epidemiological studies on hemorrhagic fever with renal syndrome in China. *J. Infect. Dis.,* **154**: 394-398.
4) Cho, H. W., and Howard, C. R. 1999. Antibody responses in humans to an inactivated hantavirus vaccine (Hantavax). *Vaccine,* **17**: 2569-2575.
5) Dekonenko, A., Yakimenko, V., Ivanov, A., Morozov, V., Nikitin, P., Khasanova, S., Dzagurova, T., Tkachenko, E., and Schmaljohn, C. 2003. Genetic similarity of Puumala viruses found in Finland and western Siberia and of the mitochondrial DNA of their rodent hosts suggests a common evolutionary origin. *Infect. Genet. Evol.,* **3**: 245-257.
6) Gavrilovskaya, I. N., Chumakov, M. P., Apekina, N. S., Ryltseva, E. V., Martiyanova, L. I., Gorbachkova, E. A., Bernshtein, A. D., Zakharova, M. A., and Boiko, V. A. 1983. Adaptation to laboratory and wild animals of the haemorrhagic fever with renal syndrome virus present in the foci of European U.S.S.R. Brief report. *Arch. Virol.,* **77**: 87-90.
7) Keay, L. 1975. Autoclavable low cost serum-free cell culture media. The growth of L cells and BHK cells on peptones. *Biotechnol. Bioeng.,* **17**: 745-764.
8) Kim, G. R., Lee, Y. T., and Park, C. H. 1994. A new natural reservoir of hantavirus: isolation of hantaviruses from lung tissues of bats. *Arch. Virol.,* **134**: 85-95.
9) Kitamura, T., Morita, C., Komatsu, T., Sugiyama, K., Arikawa, J., Shiga, S., Takeda, H., Akao, Y., Imaizumi, K., Oya, A., Hashimoto, N., and Urasawa, S. 1983. Isolation of virus causing hemorrhagic fever with renal syndrome (HFRS) through a cell culture system. *Jpn. J. Med. Sci. Biol.,* **36**: 17-25.
10) Klempa, B., Schmidt, H. A., Ulrich, R., Kaluz, S., Labuda, M., Meisel, H., Hjelle, B., and Kruger, D. H. 2003. Genetic interaction between distinct Dobrava hantavirus subtypes in *Apodemus agrarius* and *A. flavicollis* in nature. *J. Virol.,* **77**: 804-809.
11) Klempa, B., Stanko, M., Labuda, M., Ulrich, R., Meisel, H., and Kruger, D. H. 2005. Central European Dobrava Hantavirus isolate from a striped field mouse (*Apodemus agrarius*). *J. Clin. Microbiol.,* **43**: 2756-2763.
12) Klempa, B., Tkachenko, E. A., Dzagurova, T. K., Yunicheva, Y. V., Morozov, V. G., Okulova, N. M., Slyusareva, G. P., Smirnov, A., and Kruger, D. H. 2008. Hemorrhagic fever with renal syndrome caused by 2 lineages of Dobrava hantavirus, Russia. *Emerg. Infect. Dis.,* **14**: 617-625.
13) Klingstrom, J., Hardestam, J., and Lundkvist, A. 2006. Dobrava, but not Saaremaa, hantavirus is lethal and induces nitric oxide production in suckling mice. *Microbes. Infect.,* **8**: 728-737.
14) Kruger, D. H., Ulrich, R., and Lundkvist, A. A. 2001. Hantavirus infections and their prevention. *Microbes. Infect.,* **3**: 1129-1144.
15) Lee, H. W., Lee, P. W., and Johnson, K. M. 1978. Isolation of the etiologic agent of Korean Hemorrhagic fever. *J. Infect. Dis.,* **137**: 298-308.
16) Lee, H. W., and van der Groen, G. 1989. Hemorrhagic fever with renal syndrome. *Prog. Med.*