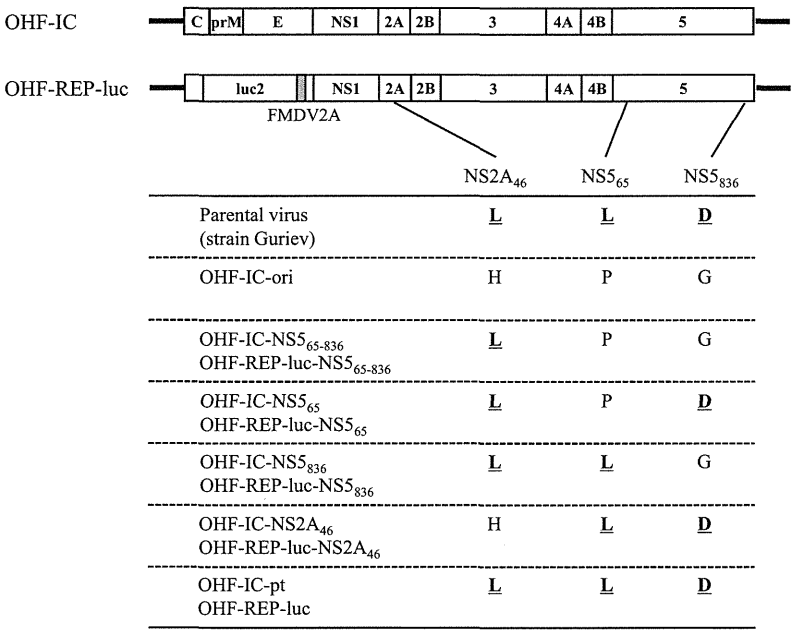

Fig. 3. Growth curves of parental Guriev, OHF-IC-pt, OHF-IC-$NS5_{65}$, OHF-IC-$NS5_{836}$, OHF-IC-$NS5_{65-836}$, and OHF-IC-$NS2A_{46}$. A Monolayer of BHK cells was infected with the individual viruses at a multiplicity of infection (MOI) of 0.01. At each time point, the media was harvested and virus titers were determined by plaque assay in BHK cells.

of the transfected cells, and a cytopathic effect was observed in these cell cultures. However no infectious virus could be recovered from cells transfected with RNAs from OHF-IC-ori, and no virus antigens were observed in the cells by immunofluorescence assay (Fig. 3B). These data indicated two things: the production of viral proteins in the cells transfected with RNAs from OHF-IC-pt was not merely the result of translation of the input RNA, and the mutations in OHF-IC-ori affected the production of viral proteins and infectious viruses.

The growth properties of OHF-IC-pt derived virus and wild-type OHFV were analyzed by monitoring the release of virus after infection. BHK cells were infected at m.o.i. of 0.01 with OHFV. Virus was harvested at 24 h intervals and the yield was quantified by plaque assay (Fig. 4). There was slight difference in the yields of virus at 24 h post-infection, but the resulting growth curves indicate similar growth properties between the parent virus and recombinant virus from OHF-IC-pt. The slight difference at 24 h post-infection may derive from the quasispecies of the parent virus stock because the virus was not plaque purified.

3.3. Characterization of NS mutant

To determine how the mutations in OHF-IC-ori affected the viral production from the OHFV infectious cDNAs, we prepared several infectious cDNAs that incorporated the mutations identified in OHF-IC-ori (Fig. 2). The OHF-IC-$NS5_{65-836}$, OHF-IC-$NS5_{65}$ and OHF-IC-$NS5_{836}$ plasmids have the NS5 mutations, and OHF-IC-$NS2A_{46}$ has the NS2A mutation. Recombinant viruses were recovered from these infectious cDNAs, and the growth curves were generated as described above.

No significant differences were observed between the growth curves of OHF-IC-pt and OHF-IC-$NS5_{65}$ (Fig. 4), indicating that the amino acid difference at NS5 position 65 (L to P) did not affect virus growth in BHK cells. Meanwhile, the growth of OHF-IC-$NS2A_{46}$, OHF-IC-$NS5_{65-836}$ and OHF-IC-$NS5_{836}$ was restricted relative to OHF-IC-pt and OHF-IC-$NS5_{65}$. These results indicate that the mutations at NS2A position 46 (L to H) and NS5 position 836 (D to G) limited the virus growth in cell culture.

To further investigate the effect of the NS2A and NS5 mutations on virus production, we prepared a replicon of OHFV containing the luciferase reporter gene with or without the NS mutations (see Section 2 and Fig. 2). These replicon RNAs were transfected into BHK cells, and luciferase activities were measured at 6 h and 3 days post-transfection. There was no difference between the luciferase activities of the replicons at 6 h post-transfection (Fig. 5A), indicating that the mutations had no effect on the initial translation of reporter gene from transfected replicon RNA. At 3 days post-transfection, the luciferase activity was lower in the lysate from the replicons with mutations at NS2A position 46 or NS5 position 836 at 37 °C (Fig. 5B). In contrast, there was no decrease in luciferase activities was detected in the lysates from the replicon with and without the mutation at NS5 position 836 at 30 °C. Luciferase activity in cells transfected with the replicon containing the NS2A-46 mutation remained diminished in cells incubated at 30 °C. Similar temperature sensitivity of NS5-836 was observed in the virus production at 30 °C (supplementary Fig. 1). These data indicate that the mutations at NS2A-46 and NS5-836 reduce viral replication by reducing RNA replication and that the NS5-836 mutation produces a temperature-sensitive (ts) defect in RNA replication.

3.4. Pathogenicity of infectious clone-derived viruses in mouse model

In our previous studies, we described that laboratory mice infected with OHFV showed clinical signs and pathology similar to reports of human infection (Holbrook et al., 2005; Tigabu et al., 2009). The pathogenicity of recombinant viruses was examined in the mouse model. Five adult C57BL/6 mice were infected subcutaneously with 1000 p.f.u. of each virus and survival was recorded for 28 days (Fig. 6).

OHF-IC-pt virus which is genetically identical to parental OHFV Guriev strain produced 100% mortality of mice (mean survival

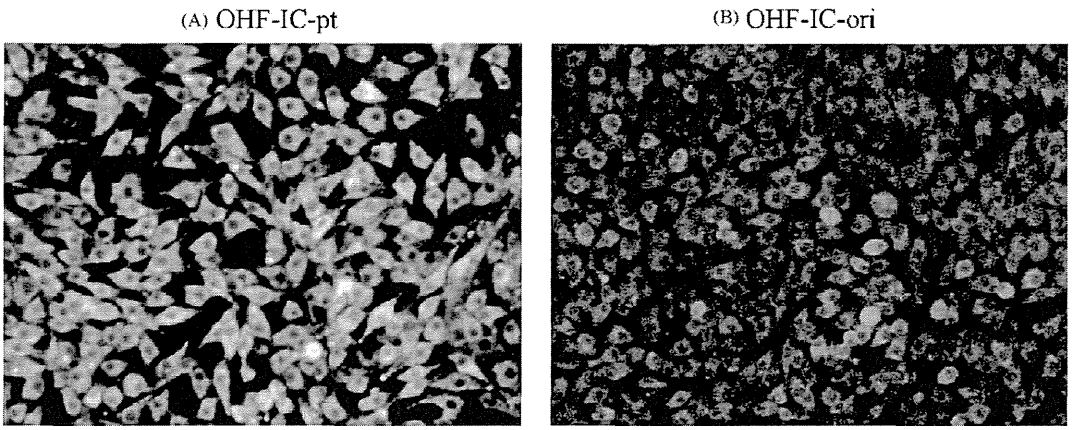

Fig. 4. Schematic representation of the genome of OHFV showing all of the amino acid coding difference between strain Guriev and constructed full-length clones (OHF-IC) and OHFV replicons used to analyze the effect of NS2A and NS5 mutations. Bold type has been used to designate the amino acids of the consensus sequence in Guriev (see text and Table 1).

time 13.8 ± 1.92). All mice showed general signs of illness such as hunched posture, ruffled fur, and general malaise, but did not have significant or consistent indications of neurologic disease. After onset of disease, they showed a sharp decrease in body weight beginning at 9 and 10 dpi. When examined postmortem, all mice had conjunctival suffusion with crusting and some had bowel hemorrhage. These results were consistent with our previous data in which mice infected with parental OHFV Guriev strain had viscerotropic disease with limited signs of neurological disease (100% mortality and mean survival time 12.8 ± 2.49) (Tigabu et al., 2009).

The OHF-IC-NS5$_{65}$ virus had similar virulence to the OHF-IC-pt virus (100% mortality and mean survival time 14.2 ± 1.92) indicating that the amino acid difference at NS5 position 65 did not affect the biological properties of OHFV confirming observations from *in vitro* studies. However, the OHF-IC-NS2A$_{46}$, OHF-IC-NS5$_{65-836}$ and OHF-IC-NS5$_{836}$ virus showed decreased virulence. The OHF-IC-NS5$_{65-836}$ and OHF-IC-NS5$_{836}$ virus killed three mice, but survival time was increased while the OHF-IC-NS2A$_{46}$ virus was completely attenuated. The NS5-836 mutation was retained in the dead mice inoculated with The OHF-IC-NS5$_{65-836}$ and OHF-IC-NS5$_{836}$ virus. All surviving mice inoculated with the OHF-IC-NS2A$_{46}$, OHF-IC-NS5$_{65-836}$ and OHF-IC-NS5$_{836}$ virus showed no signs of illness and no weight loss. These results indicate that the mutations at NS2A position 46 and NS5 position 836 affect OHFV virulence in association with the lower viral replication. All surviving mice inoculated with OHF-IC-NS2A$_{46}$, OHF-IC-NS5$_{65-836}$ and OHF-IC-NS5$_{836}$ had neutralizing antibody against OHFV (≥320 in 50% reduction), indicating that the virus was able to replicate at the initial stage of infection without causing a disease.

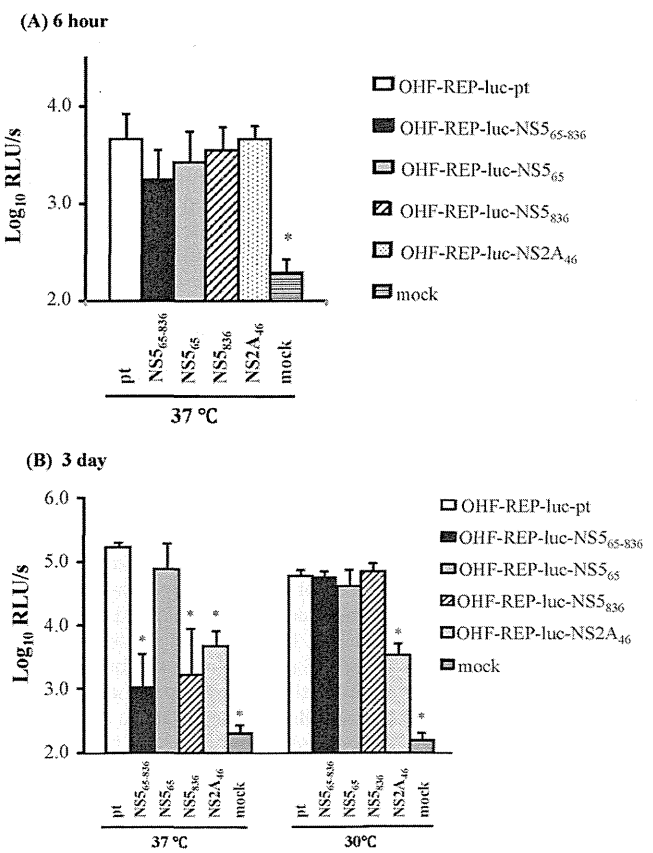

Fig. 5. Effect of NS mutations on OHFV RNA replication. BHK cells were transfected with luciferase-expressing OHFV subgenomic replicon RNAs (OHF-REP-luc) with or without the NS2A or NS5 mutations, and incubated at 37 °C or 30 °C. Luciferase activities were measured at 6 h (A) 72 h (B) post-transfection. Luciferase activities are expressed in Raw Light Units (RLU). Asterisks show the statistically significant difference compared to OHF-REP-luc-pt by Student *T* test ($P < 0.05$).

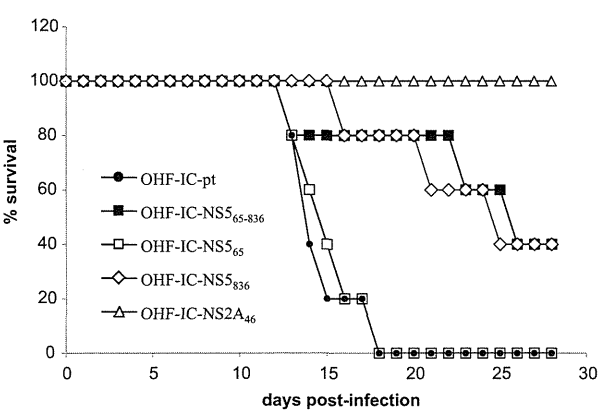

Fig. 6. Survival of mice inoculated with OHF-IC. Mice were inoculated subcutaneously with 1000 p.f.u. of OHF-IC-pt, OHF-IC-NS5$_{65}$, OHF-IC-NS5$_{836}$, OHF-IC-NS5$_{65-836}$, and OHF-IC-NS2A$_{46}$.

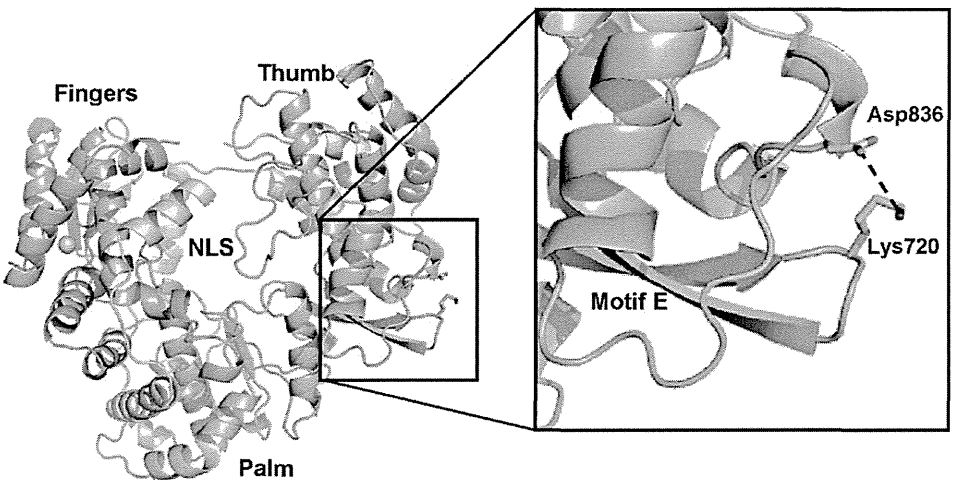

Fig. 7. Location of amino acid substitutions at Asp836 on a homology model of the OHFV RNA-dependent RNA polymerase domain in NS5 based on that of dengue virus (PDB 2J7W). The zoomed region in the right panel shows the proposed interaction of charged side chains between Asp836 and Lys720.

4. Discussion

This is the first report of generation of an infectious clone of OHFV. Infectious clones can be a valuable tool for studying the molecular biology of virus replication, virus structure, virulence determinants, and vaccine development. There is a common difficulty in the construction of full-length clones of flaviviruses, because the plasmids containing a full-length cDNA of these viruses are often unstable during propagation in *E. coli*. Therefore, by using low-copy-number plasmids and specific bacterial hosts, stable full-length infectious clones have been developed for several flaviviruses (Gritsun and Gould, 1998; Hayasaka et al., 2004; Kinney et al., 1997; Mandl et al., 1997; Shi et al., 2002; Yamshchikov et al., 2001; Yun et al., 2003). In this study, the low-copy-number plasmid pACNR was used for the construction of the full-length OHFV cDNA. This vector has been used to construct stable infectious clones of several flaviviruses and pestiviruses (Bredenbeek et al., 2003; McElroy et al., 2005; Mendez et al., 1998; Ruggli et al., 1996). The OHF-IC plasmids were stable during passaging in bacteria, indicating that this infectious clone can be useful for genetic manipulations.

The Guriev strain, which was isolated from human blood, was selected to construct a full-length infectious clone. We previously demonstrated the similarity between human and murine infection with OHFV (Holbrook et al., 2005; Tigabu et al., 2009). The OHFV-infected mice had no indication of neurological problems, and had conjunctival suffusion that has also been reported in human cases. The recombinant virus which is genetically identical to the parental Guriev strain showed similar biological properties to the parental virus, including growth kinetics and virulence characteristics. These results indicate that an efficient reverse genetics system has been established for OHFV.

Our results have identified two amino acid codon substitutions associated with attenuation of the virus production, i.e., Leu46 to His in the NS2A, and Asp836 to Gly in NS5. These substitutions decreased the efficiency of RNA replication, leading to limited virus propagation and decreased virulence in mice.

Flavivirus NS2A is a small, hydrophobic, membrane associated protein involved in RNA replication. It was reported that NS2A binds with high specificity to the 3′ untranslated region (UTR) of viral genomic RNA and to other components of the replication complex (Mackenzie et al., 1998). In addition, NS2A is considered to play roles in modulating the host-antiviral interferon response (Liu et al., 2004, 2006, 2005; Munoz-Jordan et al., 2003) and assembly/secretion processes of virus particles (Kummerer and Rice, 2002; Leung et al., 2008; Liu et al., 2003). Although the exact membrane topology of NS2A is yet to be determined, NS2A of OHFV has been predicted to span the membrane of the endoplasmic reticulum five times by several transmembrane domain prediction programs (TMHMM (Krogh et al., 2001), and TMpred (Hofmann and Stoffel, 1993)). The Leu46 residue in NS2A is located in the conserved hydrophobic residues of the predicted 2nd transmembrane region. It is possible that the Leu46 to polar His substitution affects the membrane spanning domain and the interaction with other transmembrane domains of NS2A or other membrane-associated viral proteins. A change in membrane associated protein topology could lead to a defect in the replication properties of NS2A such as the formation of the replication complex by binding with viral RNA and other components of the replication complex.

NS5 is the largest (104 kDa) of the flavivirus proteins, and three functional domains have been identified in NS5: a *S*-adenosylmethionine methyltransferase-like domain in the N-terminal region (Egloff et al., 2002; Koonin, 1993; Ray et al., 2006), a centrally located nuclear localization sequence (NLS) (Forwood et al., 1999; Kapoor et al., 1995), and an RNA-dependent RNA polymerase (RdRp) domain in the C-terminal region (Bartholomeusz and Wright, 1993; Koonin, 1991). The Asp836 residue in NS5 is located in the RdRp domain. In the homology model of OHFV RdRp domain based on the crystal structure of the dengue virus RdRp domain, this residue is within the α25 helix in the Thumb domain (Yap et al., 2007). In position 836 of the NS5 RdRp domain, negatively charged amino acids aspartic acid or glutamic acid are highly conserved among most flaviviruses. The molecular model suggests that the negatively charged Asp836 might form a salt bridge with the positively charged Lys720 in Motif E of the RdRp domain (Fig. 7). Molecular mechanics calculations also showed that the Asp836 to Gly substitution significantly reduced the interaction of the residue at position 836 with the Lys720 and Motif E (supplementary Table 1). These results led to the hypothesis that the reduction of interaction due to Asp836 to Gly substitution causes structural fluctuation, especially in Motif E. Motif E forms an antiparallel β-sheet wedged between the palm domain and several α-helices of the thumb domain. In several studies, it has been shown that some of the residues in Motif E are involved in the GTP-binding site and which have essential roles in *de novo* initiation of RNA synthesis in *Flaviviridae* polymerases (Choi et al., 2004; Lai et al., 1999; Yap et al.,

2007). It is possible that residue Asp836 is important for the structural stability of Motif E, and that the structural fluctuation of Motif E caused by the Asp836 to Gly substitution leads to a reduction in the efficiency of *de novo* initiation of RNA synthesis. In general, the conformational fluctuation of proteins is associated with their temperature. Therefore, this thermal fluctuation could be a possible cause of the temperature-sensitive property observed in the replicon studies (Fig. 5). Temperature dependence has been reported at the initiation, but not elongation, phase of *de novo* RNA synthesis by dengue virus RdRp (Ackermann and Padmanabhan, 2001). These data support the involvement of the interaction between Asp836 and Motif E in *de novo* initiation of RNA synthesis.

In summary, we have constructed an infectious cDNA clone of OHFV and demonstrated the utility of this clone in the research of OHFV pathogenesis. We have also identified previously unknown mutations in NS2A and NS5 that appear to play important roles in OHFV RNA synthesis.

Acknowledgments

This work was supported by Overseas Assignment Program for Japanese Researchers in Research into Emerging and Re-emerging Infectious Diseases from The Japan Health Sciences Foundation and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan, Health Sciences Grants for Research on Emerging and Re-emerging Infectious Disease from the Ministry of Health, Labour, and Welfare of Japan, and a Western Regional Center of Excellence (U54 AI057156) Career Development award (MRH) in part.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2010.08.023.

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Journal of Virological Methods

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An efficient *in vivo* method for the isolation of Puumala virus in Syrian hamsters and the characterization of the isolates from Russia

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Article history:
Received 5 September 2010
Received in revised form
10 December 2010
Accepted 21 December 2010
Available online 28 December 2010

Keywords:
Hantavirus
Puumala virus
Hokkaido virus
Syrian hamster
Virus isolation

A B S T R A C T

Puumala virus (PUUV) and other *Arvicolinae*-borne hantaviruses are difficult to cultivate in cell culture. To isolate these hantaviruses efficiently, hantavirus nucleocapsid protein (NP)-positive but seronegative wild rodents were selected by NP-detection ELISA. Three of 68 *Myodes glareolus* captured in Samara, Russia, were NP-positive and seronegative. Syrian hamsters were inoculated with lung homogenates from NP-positive rodents for virus propagation. Virus isolation *in vitro* was carried out by inoculation of lung homogenates of NP-positive hamsters to Vero E6 cell monolayers. Two PUUV strains (Samara49/CG/2005 and Samara94/CG/2005) from *M. glareolus* were isolated in Vero E6 cells. Nucleotide and amino acid sequence identities of the S segment of these isolates to those of PUUV F-s808 from a fatal HFRS patient in Samara region were 96.7–99.3% and 99.3–100.0%, respectively. Morphologic features of Vero E6 cells infected with PUUV strain Samara49/CG/2005 were quite similar to those of Hantaan virus-infected cells. Isolation of Hokkaido virus from *Myodes rufocanus* captured in Hokkaido, Japan, was also performed. Hokkaido virus NP and RNA were recovered and maintained in hamsters. These results suggest that inoculation of Syrian hamsters with rodent samples is an efficient method for the isolation and maintenance of PUUV and other *Arvicolinae*-borne hantaviruses.

1. Introduction

Hantaviruses are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS; Krüger et al., 2001; Schmaljohn and Hjelle, 1997). Rodents and *Soricomorpha* species are the natural reservoirs of these viruses, and humans acquire infection by inhaling the excreta of infected animals. Many rodent-borne hantaviruses are known to be pathogenic to humans, and although large numbers of hantaviruses have been identified in *Soricomorpha* species, thus far, no relationship between *Soricomorpha*-borne hantaviruses and human disease have been reported (Song et al., 2007a). Hantaviruses are classified in the genus *Hantavirus*, within the family *Bunyaviridae*, and possess a genome composed of three negative-stranded RNA segments. The small (S), medium (M), and large (L) genome segments encode nucleocapsid protein (NP), two glycoproteins (Gn, Gc), and RNA polymerase, respectively (Schmaljohn and Nichol, 2001).

Each rodent-borne hantavirus has its own host, and the genus *Hantavirus* contains at present more than 20 species (Nichol et al., 2005). These viruses are divided into three large groups by host animal classification: the *Murinae*-borne, *Arvicolinae*-borne, and *Sigmodontinae*- or *Neotominae*-borne hantaviruses. Among *Arvicolinae*-borne viruses, Puumala virus (PUUV; Tkachenko et al., 1984) is well known as the causative agent of HFRS in European countries and Russia. Approximately 10,000 clinical cases of HFRS annually occur in Europe and western Russia, caused primarily by PUUV (Kariwa et al., 2009; Tkachenko et al., 1999; Vapalahti et al., 2003). The natural host of PUUV is *Myodes glareolus*, a rodent dis-

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doi:10.1016/j.jviromet.2010.12.019

tributed widely in Europe and Russia. PUUV infects *M. glareolus* persistently and humans acquire the infection by inhaling rodent excreta or after being bitten by infected rodents (Hardestam et al., 2008; Schmaljohn and Hjelle, 1997). PUUV genome sequence data have accumulated and the viruses have been divided into several genetic linages (Sironen et al., 2001), but few studies have examined the biological properties of PUUVs of different lineages.

In Far East Asia, Hokkaido virus (HOKV) is carried by *Myodes rufocanus* (Kariwa et al., 1995; Plyusnina et al., 2008; Zhang et al., 2010; Zou et al., 2008). HOKV is related serologically to, but distinct from, PUUV (Kariwa et al., 1999). However, HOKV has not yet been isolated from its natural reservoir, and no HFRS cases have been linked definitively to HOKV infection (Kariwa et al., 2000).

Because of its slow growth in cell culture, the isolation of *Arvicolinae*-borne hantaviruses is believed to be more difficult than in other rodent-borne hantaviruses, which has hampered at virus characterization. Here this report shows an efficient method for isolating hantaviruses via inoculation of NP-positive samples to Syrian hamsters. Two PUUV strains were isolated from *M. glareolus* captured in Samara region of Russia, and the genetic, antigenic, and morphological properties of these isolates were analyzed. In addition, HOKV NP and RNA were recovered successfully in Syrian hamsters from *M. rufocanus*.

2. Materials and methods

2.1. Cells and medium

Vero E6 cells (ATCC No. CRL-1586; American Type Culture Collection, Manassas, VA) were maintained in minimum essential medium with Eagle's salts (MEM; Invitrogen, Carlsbad, CA) supplemented with fetal bovine serum (10%; MP Biochemicals, Aurora OH), L-glutamine (2 mM; Kanto Chemical, Tokyo, Japan), penicillin G (100 IU/ml; Meiji Seika, Tokyo, Japan), and streptomycin (100 μg/ml; Meiji Seika).

2.2. Rodent capture and sampling

Sixty-eight *M. glareolus* were captured in the Samara region of Russia in 2005 (Kariwa et al., 2009), and 48 *M. rufocanus* at Nakagawa town, Hokkaido Prefecture, Japan, in 2004 (Abu Daud et al., 2007). Lungs, kidneys, spleen, liver, blood clot, and serum were collected from captured animals.

2.3. Antibodies

PUUV strain Sotkamo-infected mouse serum, anti-HOKV recombinant NP (rNP) rabbit IgG, and anti-NusA rabbit IgG were used to detect hantavirus NP (Abu Daud et al., 2007; Lokugamage et al., 2003). In addition, anti-hantavirus monoclonal antibodies (mAbs) were used for the antigenic characterization of hantaviruses (Lundkvist and Niklasson, 1992; Lundkvist et al., 1996a,b, 2002; Yoshimatsu et al., 1996). Anti-PUUV mAbs were provided kindly by Dr. A. Lundkvist.

2.4. Indirect immunofluorescent antibody assay (IFA)

For the detection of anti-hantavirus antibodies in wild rodents, IFA was carried out using a protocol described previously (Kariwa et al., 2009). Vero E6 cells were infected with PUUV strain Sotkamo, Hantaan virus strain 76-118 (HTNV; Lee et al., 1978) or Seoul virus strain SR-11 (SEOV; Kitamura et al., 1983), and infected cells were fixed with cold acetone on 24-well slides. Wild rodent sera were spotted onto the slides and incubated at 37 °C for 1 h. Slides were then washed with phosphate-buffered saline (PBS) three times and Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen) was applied. After incubation for 1 h and washing, the cells were observed under a fluorescence microscope. Scattered and fine granular fluorescence in the cytoplasm of Vero E6 cells was considered as a positive reaction. For detecting hantaviral antigen in Vero E6 cells, inoculated cells on 24-well slides were stained with mAb E5/G6 to hantavirus NP (Yoshimatsu et al., 1996) and Alexa Fluor® 488 goat anti-mouse IgG.

2.5. Hantavirus NP detection

Hantavirus NP was detected by ELISA (NP-ELISA), as described previously (Abu Daud et al., 2007). Briefly, lungs of wild rodents were homogenized in lysis buffer [0.01 M Tris–HCl (pH 7.8), 2% Triton-X, 0.15 M NaCl, 0.6 M KCl, 5 mM EDTA, aprotinin 2 μg/ml, leupeptin 5 μl/ml, pepstatin 2 μg/ml, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Homogenates were kept on ice for 30 min and centrifuged at 4000 × *g* for 10 min. Supernatants were stored at −80 °C. Ninety-six-well EIA/RIA plates (Corning, Corning, NY) were coated with anti-rNP rabbit IgG (2 μg/ml) in carbonate/bicarbonate buffer (Sigma–Aldrich, St. Louis, MO) at 4 °C overnight. Plates coated with anti-NusA rabbit IgG were used as a control. The plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked with Block Ace (Dai Nippon Pharmaceutical, Osaka, Japan) at 37 °C for 1 h. Lung homogenates in the lysis buffer (10%) were diluted with PBST (1:4) and added to the plates, followed by incubation and washing. Anti-PUUV strain Sotkamo mouse serum in PBST (1:1000 dilution) was then added and plates were incubated as described above. After washing, goat anti-mouse IgG horseradish peroxidase conjugate (1:10,000 dilution; Jackson ImmunoResearch, West Grove, PA) was added and plates were incubated as before. *O*-phenilenediamine (100 μl; Sigma–Aldrich) with hydrogen peroxide was added to each well, and plates were left at room temperature for 30 min. The optical densities (ODs) of wells coated with anti-rNP rabbit IgG minus those of wells coated with anti-NusA rabbit IgG were then calculated.

2.6. Virus isolation

2.6.1. Inoculation of Syrian hamsters with lung homogenates

The samples for inoculation were selected using IFA and NP-ELISA results. Lung tissues from antibody-negative and NP-positive wild rodents were inoculated into Syrian hamsters. Lungs of *M. glareolus* and *M. rufocanus* were homogenized using a cold pestle, mortar, and sea sand in MEM. Homogenates (10%) were centrifuged at 2000 × *g* for 5 min and the supernatants were inoculated subcutaneously (0.1 ml) to Syrian hamsters (4-week-old, male; Japan SLC, Shizuoka, Japan). Twelve days post-inoculation (d.p.i.), hamsters were killed by cardiac puncture under anesthesia using sevoflurane, and lung, kidney, spleen, and serum samples collected. All animal experiments using Syrian hamsters were performed according to the guidelines of animal experimentation at the School of Veterinary Medicine, Hokkaido University, and carried out in a Biosafety level 3 animal facility.

2.6.2. Inoculation of Vero E6 cells with lung homogenates

Lung homogenates of hamsters were centrifuged at 2000 × *g* for 5 min and supernatants were used to inoculate Vero E6 cells by centrifugation at 670 × *g* for 1 h at room temperature (RT; Kariwa et al., 1994). Inocula were discarded, and cells incubated at 37 °C in a 5% CO_2 atmosphere. Cells were subcultured at 14-day intervals. At subculture, a proportion of cells were collected, spotted on 24-well glass slides, and incubated at 37 °C for 4 h in 5% CO_2. Slides were fixed and subjected to IFA. The presence of hantaviral RNA in collected cells was assessed by reverse-transcription polymerase chain reaction (RT-PCR: Abu Daud et al., 2007; Kariwa et al., 2009).

2.7. Sequencing hantavirus genes

Total RNA of hantavirus-infected Vero E6 cells was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). RNA was reverse-transcribed by SuperScript II (Invitrogen) and random primers (Invitrogen) according to the manufacturer's instructions. The 3′ ends of viral sense or antisense cDNA were dCTP-tailed using the 5′ RACE System for Rapid Amplification of cDNA Ends (Ver. 2.0; Invitrogen). The cDNA was amplified using an abridged anchor primer (Invitrogen) and PUUV-specific primers. PCR products were extracted and purified from agarose gel with a Wizard® SV Gel and PCR Clean-up System (Promega, Fitchburg, WI) and sequenced directly using a BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.8. Genetic analysis

Hantavirus nucleotide and amino acid sequences were compared using Genetyx-mac ver. 10.0 (Genetyx, Tokyo, Japan). Sequence data of PUUV strains Kazan (GenBank Accession Nos. Z84204, Z84205, and EF405801), DTK/Ufa97 (AB297665, AB297666, and AB297667), CG1820 (M32750, M29979, and M63194), and Sotkamo (NC_005224, NC_005223, and NC_005225) were downloaded from the NCBI Web site (http://www.ncbi.nlm.nih.gov/) and used for comparison.

2.9. Electron microscopy

Newly isolated hantaviruses were inoculated to Vero E6 cells and incubated for 14 days. Infected cells were harvested and pre-fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (CB; pH 7.4) for 2 h at 4 °C. Pre-fixed cells were post-fixed with 1% OsO_4 in CB for 1 h at room temperature, dehydrated with an ethanol series, and then substituted with QY-1 (Nisshin EM, Tokyo, Japan) three times for 1 h at room temperature. After substituting, cells were embedded in Epon812 resin for 2 days at 60 °C. Ultrathin sections were cut using an Ultracut S ultramicrotome (Reichert-Nissei, Vienna, Austria) and mounted on nickel grids. Sections were then stained with saturated aqueous uranyl acetate and lead citrate and examined under a JEM-1210 electron microscope (JEOL, Tokyo, Japan) at 80 kV.

2.10. Immunoelectron microscopy

Vero E6 cells infected with hantavirus were fixed with 2 glutaraldehyde and 2% paraformaldehyde in 0.1 M CB for 2 h at 4 °C. After dehydration in an ethanol series, cells were substituted with 1:1 ethanol to Lowicryl K4M resin (TAAB Laboratories Equipment, Berkshire, UK) and cells were embedded in Lowicryl K4M resin for 2 days at −20 °C and then at room temperature for 2 days under UV light. Ultrathin sections were rinsed with PBS and treated with 10% bovine serum albumin for 1 h at room temperature. Sections were then incubated with anti-HOKV rNP rabbit IgG overnight at 4 °C, rinsed and incubated with colloidal gold (10 nm)-labeled anti rabbit IgG (EY Laboratories, San Mateo, CA) for 1–2 h at room temperature. Sections were then rinsed and finally stained with uranyl acetate and lead citrate at room temperature.

3. Results

3.1. Detection of hantavirus NP by NP-ELISA in wild rodents

Culture supernatants of Vero E6 cells infected with PUUV strain Sotkamo were subjected to NP-ELISA. PUUV NP was present in a dose-dependent manner (Fig. 1). Ten lung homogenate samples of normal Syrian hamsters were applied to NP-ELISA. The mean

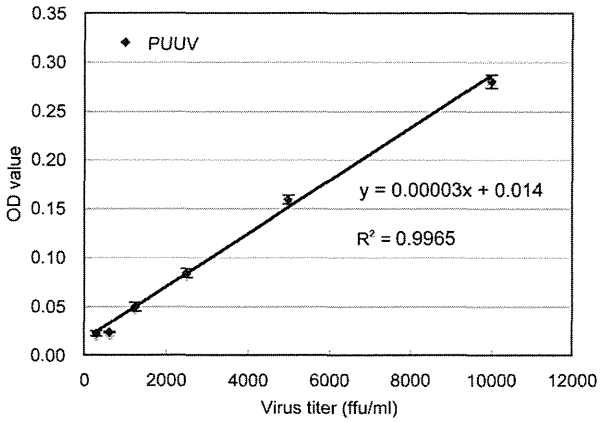

Fig. 1. Detection of PUUV NP by NP-ELISA in a dose-dependent manner. Culture fluid of Vero E6 cells infected with PUUV Sotkamo strain was collected and subjected to NP-ELISA. The standard was calculated by the least-squares method.

OD value and standard deviation (SD) were −0.01085 and 0.011, respectively. The cutoff value was determined at 0.025 by the mean OD value plus 3.25 times SD ($p < 0.01$) of normal hamsters. Five of 68 *M. glareolus* and two of 48 *M. rufocanus* were hantavirus NP-positive (Table 1). All NP-positive rodents were also RNA-positive. Six of 68 *M. glareolus* and five of 48 *M. rufocanus* were seropositive. Three of five NP-positive *M. glareolus* and two of two NP-positive *M. rufocanus* were seronegative. Two *M. glareolus* were positive for both NP and antibodies. Most seropositive rodents (9/11) were also RNA-positive. Three *M. glareolus* (S6, S49, and S94) and two *M. rufocanus* (N40 and N55) lung samples, which were NP and RNA-positive but seronegative, were selected for virus isolation.

3.2. Isolation of PUUV

Syrian hamsters were inoculated subcutaneously with *M. glareolus* lung homogenates. At 12 days d.p.i., all hamsters were killed

Table 1
Detection of anti-hantavirus antibodies, NP and virus RNA in *M. glareolus* ($n = 68$) and *M. rufocanus* ($n = 48$).

No. of rodents	Species	Antibodies	NP ELISA[a]		RT-PCR
		IFA titer	OD value	Result	
S6[b]	***M. glareolus***[c]	**<16**	**0.417**	**+**	**+**
S49		**<16**	**0.412**	**+**	**+**
S94		**<16**	**0.464**	**+**	**+**
S112		512	0.063	+	+
S128		256	0.195	+	+
S68		1024	−0.001	−	+
S147		1024	0.001	−	+
S66		256	0.003	−	−
S137		512	−0.004	−	−
Others ($n = 59$)		<16	<0.006	−	−
N40	***M. rufocanus***[d]	**<16**	**0.566**	**+**	**+**
N55		**<16**	**0.455**	**+**	**+**
N10		256	−0.031	−	+
N13		512	−0.025	−	+
N49		1024	−0.015	−	+
N73		1024	−0.010	−	+
N89		1024	−0.010	−	+
Others ($n = 41$)		<16	<−0.001	−	−

[a] The cut-off point of NP-ELISA was 0.025 OD value.
[b] Rodent samples written in bold characters were used for virus isolation.
[c] A total of 68 *M. glareolus* were captured in Samara region, Russia in 2005.
[d] A total of 48 *M. rufocanus* were captured in Nakagawa Town, Hokkaido, Japan in 2004.

Table 2
Detection of hantavirus from Syrian hamsters injected with lung homogenate of *M. glareolus* and *M. rufocanus*.

No. of rodents	Species	Hamster No.	Antibodies	NP-ELISA[a]		RT-PCR	Virus replication in Vero E6 cell
			IFA titer	OD value	Result		
S6	*M. glareolus*	S6-1	<16	0.001	–	–	–
		S6-2	<16	0.052	+	+	–
		S6-3	<16	−0.014	–	–	N.D[b]
S49	*M. glareolus*	S49-1	<16	0.001	–	–	–
		S49-2	<16	0.082	+	+	N.D
		S49-3	<16	0.322	+	+	+
S94	*M. glareolus*	S94-1	<16	0.114	+	+	+
		S94-2	<16	−0.042	–	–	N.D
		S94-3	<16	−0.013	–	–	N.D
N40	*M. rufocanus*	N40-1	<16	0.009	–	–	–
		N40-2	<16	−0.016	–	–	N.D
		N40-3	<16	−0.001	–	–	N.D
N55	*M. rufocanus*	N55-1	<16	0.213	+	+	–
		N55-2	<16	0.022	–	–	–
		N55-3	<16	0.006	–	–	N.D

[a] The cut-off point of NP-ELISA was 0.025 OD value.
[b] N.D, not done.

Table 3
Successive recovery of HOKV in Syrian hamsters.[a]

Hamster No.	Inoculum	Days post inoculation	Antibodies	NP ELISA[b]		RT-PCR
			IFA titer	*OD value*	*Result*	
N55-4	Kidney homogenate of	12	<16	−0.004	–	+
N55-5	No. N55-1	12	<16	0.090	+	+

[a] Kidney homogenate of No. N55-1 hamster (Table 2) was further inoculated to Syrian hamsters. Lungs of the inoculated hamsters were collected at 12 days post inoculation.
[b] The cut-off point of NP-ELISA was 0.025 OD value.

and lungs, other organs, and sera collected. No antibodies to PUUV were detected in these animals, but some were both RNA- and NP-positive (Table 2). Lung homogenates of hamsters (S6-2, S49-3 and S94-1) were used to inoculate Vero E6 cells. At 28 d.p.i., NP and RNA of PUUV were detected in Vero E6 cells inoculated with lung samples from S49-3 and S94-1. Infectivity was present in culture supernatants. Therefore, two PUUV strains were isolated successfully and designated as Samara49/CG/2005 and Samara94/CG/2005.

3.3. Recovery of HOKV from Syrian hamsters

Syrian hamsters were inoculated with *M. rufocanus* lung homogenates and organs were collected at 12 d.p.i. HOKV RNA and NP were detected in hamster, No. N55-1 (Table 2). Lung homogenate from this hamster was used for virus isolation, but no HOKV NP was detected. Kidney homogenate of hamster N55-1 was inoculated into Syrian hamsters for recovery of HOKV (Table 3). Twelve days post-inoculation, lungs, other organs, and blood were

Table 4
Antigenic characterization of hantaviruses by a panel of monoclonal antibodies.

mAb	Immunized virus	Antigenic site[a]	PUUV/Russia			PUUV	HTNV	SEOV
			Sam49[b]	Sam94[c]	Ufa97	Sotkamo	76-118	SR-11
GBO4	PUUV Sotkamo	NP(4)	+[d]	+	–	+	+	+
3H9	PUUV Sotkamo	NP(N-a)	+	+	+	+	–	–
5E1	PUUV Sotkamo	NP(N-b)	+	+	+	+	–	–
5B5	PUUV Sotkamo	NP(N-c)	+	+	+	+	–	–
3G5	PUUV Sotkamo	NP(N-d)	+	+	+	+	–	–
5F4	PUUV Sotkamo	NP(N-e)	+	+	+	+	–	–
1C12	PUUV Sotkamo	NP(N-f1)	+	+	+	+	+	+
3E11	PUUV Sotkamo	NP(N-f1)	+	+	+	+	+	+
5A3	PUUV Sotkamo	NP(N-f3)	+	+	–	+	+	+
2E12	PUUV Sotkamo	NP(N-g)	+	+	+	+	+	+
4C3	PUUV Sotkamo	NP(N-h1)	+	+	+	+	+	+
4E5	PUUV Sotkamo	NP(N-h2)	+	+	+	+	+	+
3C11	Tula virus	NP(N-C)	+	+	+	+	–	–
1C8	Tula virus	NP(N-E)	+	+	+	+	–	–
5A2	PUUV Sotkamo	G1	+	+	+	+	–	–
5B7(Puu)	PUUV Sotkamo	G2	+	+	+	+	–	+
4G2	PUUV Sotkamo	G2	+	+	+	+	–	–

[a] Yoshimatsu et al. (1996), Lundkvist and Niklasson (1992) and Lundkvist et al. (1996a,b, 2002).
[b] Sam49: Samara49/CG/2005.
[c] Sam94: Samara94/CG/2005.
[d] Reactivities of monoclonal antibodies to various hantaviruses were analyzed by indirect fluorescent antibody assay.

Table 5
Nucleotide and amino acid identities[a] among Puumala viruses (%).

RNA segment	Strain	Strain					
		Russia/Volga					Finland
		Samara49/CG/2005	Samara94/CG/2005	Kazan	DTK/Ufa97	CG1820	Sotkamo
S	Samara49/CG/2005	–	97.0	95.4	94.5	92.5	82.8
	Samara94/CG/2005	99.3	–	95.2	93.9	92.1	83.1
	Kazan	99.1	98.4	–	94.5	92.7	83.2
	DTK/Ufa97	99.3	98.6	98.8	–	98.0	83.1
	CG1820	99.1	98.4	98.6	99.3	–	82.2
	Sotkamo	96.8	96.1	96.8	96.5	96.3	–
M	Samara49/CG/2005	–	93.4	93.5	85.1	84.8	84.0
	Samara94/CG/2005	99.0	–	93.8	85.4	85.1	83.9
	Kazan	99.2	99.0	–	85.9	85.7	84.7
	DTK/Ufa97	97.0	96.7	97.0	–	99.5	83.0
	CG1820	96.3	96.1	96.4	99.2	–	82.8
	Sotkamo	94.9	94.6	95.2	94.4	93.8	–
L	Samara49/CG/2005	–	96.7	93.8	87.5	87.5	85.1
	Samara94/CG/2005	99.4	–	93.6	87.4	87.4	85.1
	Kazan	99.6	99.5	–	87.2	87.1	85.0
	DTK/Ufa97	98.9	98.7	99.1	–	99.7	84.6
	CG1820	98.7	98.6	98.9	99.8	–	84.6
	Sotkamo	97.3	97.1	97.4	97.4	97.3	–

[a] Values to the right above the diagonal show nucleotide identities; those to the left below the diagonal show amino acid identities.

collected. One of two hamsters was both NP- and RNA-positive. Therefore, HOKV was recovered successively from Syrian hamsters.

3.4. Antigenic characterization of PUUV isolates

Antigenic properties of strain Samara49/CG/2005 and Samara94/CG/2005 were characterized by IFA using a panel of anti-hantavirus mAbs (Table 4). The majority of mAbs to NP and glycoproteins had a similar reaction pattern among PUUVs strains, including the new PUUV isolates. Although Russian strain DTK/Ufa97 (Abu Daud et al., 2008) did not react with mAbs GBO4 and 5A3, these mAbs reacted with Samara49/CG/2005 and Samara94/CG/2005. Antisera to the PUUV isolates were prepared and a cross-neutralization test was performed as described previously (Lokugamage et al., 2004). Antisera to PUUV isolates from the Samara region had similar neutralizing titers to other PUUVs (data not shown).

3.5. Sequencing and genetic analysis

The complete nucleotide sequences of all genome segments of Samara49/CG/2005 (Accession Nos. AB433843, AB433850, and AB574183) and Samara94/CG/2005 (Accession Nos. AB433845, AB433852, and AB574184) were determined (Table 5). The S, M, and L segments of these isolates were 1828, 3682, and 6550 bp long, respectively. Genome sequences of Samara49/CG/2005 and Samara94/CG/2005 were similar (S segment, 97.0%; M segment, 93.4%; L segment, 96.7%). These two strains showed higher sequence identities to Russian/Volga PUUVs such as Kazan, DTK/Ufa97, and CG1820 strains (S segment, 92.1–95.4%; M segment, 84.8–93.8%; L segment, 87.4–93.8%) than to the Finnish PUUV strain Sotkamo (S segment, 82.8–83.1%; M segment, 83.9–84.0%; L segment, 85.1%).

3.6. Electron microscopy

The ultrastructure of PUUV-infected Vero E6 cells was analyzed by transmission electron microscopy (TEM; data not shown). At least three morphological characteristics were observed in infected cells. The first was the existence of cytoplasmic inclusion bodies, which were of variable size, and their inner structure varied from round to filar. Immunoelectron microscopy demonstrated accumulation of PUUV NP within these inclusion bodies. The second morphological characteristic was the presence of virion-like structures, likely with a bilayered envelope (70–90 nm diameter). The third was the multilayered membrane of vesicles with a "rose flower"-like structure. These structures of PUUV-infected cells were similar to HTNV-infected cells. No such morphological changes were observed in uninfected Vero E6 cells.

4. Discussion

The purpose of this study was to establish an efficient method for isolating *Arvicolinae*-borne hantaviruses. PUUV, which is carried by *M. glareolus* and the causative agent of HFRS in Europe and European Russia, is known to be difficult to isolate. Although much nucleotide sequence information is available, the biological properties of PUUVs from different locations have not been characterized fully due to a lack of a sufficient number of PUUV strains.

Other *Arvicolinae*-borne hantaviruses are found in the east of the Eurasian continent, e.g., HOKV, Muju virus (MUJV), Khabarovsk virus (KHAV), Vladivostok virus (VLAV), Yuanjiang virus (YUAV) and Topogravov virus (Hörling et al., 1996; Kariwa et al., 1995, 1999; Song et al., 2007b; Vapalahti et al., 1999; Zhang et al., 2010). HOKV is carried by *M. rufocanus* in Japan, Far East Russia and northeastern China, but no disease has been reported to be associated with HOKV infection. Although HOKV was first identified in 1995, attempts at virus isolation have thus far been unsuccessful. MUJV is maintained in *Myodes regulus* in South Korea, and about 7% of patients with HFRS had antibodies with a higher affinity for PUUV than HTNV, although PUUV is not present in this region. Therefore, MUJV may be the causative agent of some HFRS in South Korea, although MUJV has not been isolated (Song et al., 2007b). KHAV, VLAV and YUAV are carried by *Microtus fortis* but are distinct genetically (Plyusnina et al., 2008; Zhang et al., 2010). These viruses are considered to be nonpathogenic to humans. KHAV, but not VLAV and YUAV, has been isolated in Vero E6 cells. Virus isolation is necessary for analysis of the biological properties of these viruses. Although Vero E6 cells are used typically for hantavirus isolation, *Arvicolinae*-borne hantaviruses produce a lower yield than other rodent-borne hantaviruses (Kariwa et al., 1994). In addition,

seropositive rodents were used for virus isolation because hantavirus infects persistently wild rodents. However, since samples from rodents infected persistently may contain neutralizing antibody, the virus may be neutralized during isolation procedures. Both hantavirus NP and higher levels of viral RNA were detected in seronegative than in seropositive infected animals (Abu Daud et al., 2007). Thus, the use of hantavirus antigen and RNA-positive, but seronegative, animals will be likely to increase the probability of successful isolation. Isolation of HOKV, or MUJV from mice, rats, and Mongolian gerbils has been attempted (Lokugamage et al., 2003; Song et al., 2007b) but all were unsuccessful. Other studies used Syrian hamsters for evaluating a PUUV vaccine (Chu et al., 1995). Preliminary data showed that Syrian hamsters are sensitive highly to PUUV (Kariwa et al., unpublished data). In this study, Syrian hamsters were used to isolate PUUV from *M. glareolus*.

To select the most appropriate sample for virus isolation, NP-ELISA was performed on lung homogenates of wild rodents (Abu Daud et al., 2007). Seronegative and viral RNA-positive rodents contained higher NP levels (Table 1), and thus NP-positive and seronegative animals were selected for inoculation into Syrian hamsters.

After inoculation of NP-positive samples, some hamsters became NP- and viral RNA-positive (Table 2). Lung homogenates of these hamsters were used to inoculate Vero E6 cells, and in this way two PUUVs (Samara49/CG/2005 and Samara94/CG/2005) were isolated from *M. glareolus* no. S49 and S94. These isolates were then characterized genetically and serologically, and compared to other PUUVs. Although PUUVs show genetic variation (Sironen et al., 2001), the Samara isolates were more related to the Russian/Volga PUUV than to the Finnish lineages genetically. Despite this genetic variability, antigenicities of PUUV lineages were conserved relatively. In addition, the S segment nucleotide sequences of these strains and that of a hantavirus from a patient with HFRS in Samara (F-s808; GenBank Accession No. AF411446) were similar (nt, 96.7–99.3%; aa, 99.3–100.0%). Thus, these strains may be pathogenic to humans. The ultrastructure of PUUV-infected cells was examined by electron microscopy (data not shown). Morphologic features of strain Samara49/CG/2005-infected Vero E6 cells were similar to those of cells infected with Hantaan virus, which is carried by *Apodemus agrarius* and is the causative agent of HFRS in Asia (Hung et al., 1985; Xu et al., 2007). Although Sin Nombre virus, which is carried by *Peromyscus maniculatus* and the causative agent of HPS in North America, matures on the plasma membrane (Goldsmith et al., 1995), no such structures were observed in PUUV-infected cells.

Unfortunately, isolation of HOKV in Vero E6 cells was unsuccessful. The HOKV genome was detected in HOKV-inoculated Vero E6 cells at 14 and 28 d.p.i., but not after 42 and 56 d.p.i. (data not shown). These results indicate that HOKV can infect, but cannot replicate in Vero E6 cells. HOKV-positive hamster tissue was then inoculated into hamsters for recovery and maintenance of HOKV. HOKV viral RNA was detected in all and HOKV NP in one of two hamsters (Table 3). These results suggest that the Syrian hamster is an efficient animal species for HOKV recovery from *M. rufocanus*. Use of suckling hamsters may increase the efficacy of HOKV recovery (Chu et al., 1995).

In conclusion, inoculation of samples from NP-positive and seronegative rodents into Syrian hamsters is an efficient method for isolating PUUV. This strategy may be also applied to recovery and isolation of other *Arvicolinae*-borne hantaviruses.

Acknowledgments

All of the people who worked in the field in the Samara Region were thanked for assistance with rodent trapping and supported this survey. This study was supported financially by Grants-in-Aid for Scientific Research (16405034 and 17255009) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and by a Health and Labour Sciences Research Grant on Emerging and Re-emerging Infectious Diseases from the Japanese Ministry of Health, Labour and Welfare. This study was also supported by the global COE Program for Zoonosis Control (Hokkaido University).

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Microbiology and Immunology

Microbiol Immunol 2011; **55**: 100–107
doi:10.1111/j.1348-0421.2010.00296.x

ORIGINAL ARTICLE

Development of an ELISA system for tick-borne encephalitis virus infection in rodents

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ABSTRACT

Tick-borne encephalitis (TBE) virus causes severe encephalitis with serious sequelae in humans. An epizootiological survey of wild rodents is effective to detect TBE virus-endemic areas; however, limited serological diagnostic methods are available to detect anti-TBE virus antibodies in wild rodents. In this study, ELISAs for the detection of rodent antibodies against the TBE virus were developed using two recombinant proteins, domain III of the E protein (EdIII) and subviral particles (SPs), as the antigens. As compared with the neutralization test, the ELISA using EdIII had 77.1% sensitivity and 80.0% specificity, and the ELISA using SPs had 91.4% sensitivity and 100% specificity. Furthermore, when the ELISAs were applied to the epizootiological survey in the TBE virus-endemic area, both of the ELISAs was able to detect wild rodents with TBE virus-specific antibodies. This is the first study to show that ELISAs using recombinant antigens can be safe and useful in the detection of TBE virus-infected wild rodents in epizootiological research.

Key words E protein, epizootiology, rodents, subviral particles, tick-borne encephalitis.

The tick-borne encephalitis (TBE) virus, which belongs to the genus *Flavivirus* within the family *Flaviviridae*, causes severe encephalitis with serious sequelae in humans (1). The TBE virus occurs widely across Europe, Russia and Far-Eastern Asia, including Japan (2–6), and more than 10 000 cases of the disease are reported annually. The TBE virus has been subdivided into three subtypes: the far-eastern subtype known to cause Russian spring-summer encephalitis in Russia, the western European subtype known to cause Central European encephalitis in many European countries, and the Siberian subtype. The TBE virus has a significant impact on public health in the endemic regions.

The prevalence of the TBE virus in nature depends on the transmission cycles of the interactions among the viruses, their vector ticks and their vertebrate hosts (7). The *Ixodes* ticks are primary tick vectors that play a crucial role in maintaining the transmission cycle of the TBE virus. The major tick vector for the far-eastern subtype and the Siberian subtype is *Ixodes persulcatus* and that for the western European subtype is *I. ricinus*. The most important vertebrate hosts for the TBE virus are rodents that have the highest population densities within an endemic focus (generally *Apodemus*, *Clethrionomys* or *Microtus* species).

For the control of the TBE virus infection, it is important to specify the TBE virus-endemic area and design an effective vaccination plan. An epizootiological survey of field rodents is effective in the detection of TBE virus-endemic areas; however, limited serological diagnostic

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Received 29 September 2010; revised 24 November 2010; accepted 25 November 2010.

List of Abbreviations: ALP, alkaline phosphatase; DDW, deionized, distilled water; EdIII, domain III of the E protein; OD, optical density; PBST, phosphate-buffered saline containing 0.05% Tween 20; prM, precursor M; P–N, positive–negative difference; RSSE, Russian spring-summer encephalitis; SP, subviral particle; TBE, tick-borne encephalitis.

methods are available to detect anti-TBE virus antibodies in wild rodents. The neutralization test is the most specific serological test of TBE virus infection, but it has several disadvantages. Since the TBE virus is classified as a biosafety level 3 or 4 virus, a high-level biocontainment facility is required to handle the live virus in the neutralization test. The neutralization test takes several days for the diagnosis and it is not effective to handle many samples at once. Therefore, safe and simple serological diagnostic methods for wild rodents are required for epizootiological surveys.

Flavivirus virions are 40–50 nm in diameter, spherical in shape and contain a nucleocapsid and an envelope (8). The flavivirus envelope has two proteins, M and E. The E protein mediates virus entry via receptor-mediated endocytosis and also carries the major antigenic epitopes leading to a protective immune response (9). X-ray crystallographic resolution of the structure of the E ectodomain of the TBE virus revealed that the E protein consists of three domains (domains I, II, III) and forms head-to-tail homodimers that lie parallel to the viral envelope (10). Domain III of the E protein is considered to play an important role in receptor binding and to have the major epitopes to neutralizing antibodies (11). In several flaviviruses, domain III expressed as recombinant proteins has been used as an antigen for serological diagnosis (12–14). Furthermore, it has been shown that the co-expression of precursor M (prM) and E proteins lead to the production of subviral particles (SPs) (15). The SPs are smaller particles than authentic virions, but the antigenicity and immunogenicity of the SPs are similar to those of the native virus (16); therefore, the SPs are used as the antigen for serological diagnosis and vaccines (17–20). These recombinant proteins can be used as safe and useful substitutions for infectious viruses in serological diagnosis.

In this study, ELISAs for the detection of rodent antibodies against the TBE virus were developed using two recombinant proteins, domain III of the E protein and SPs, as the antigens. The ELISAs were evaluated using the serum samples of TBE virus-infected wild rodents in Hokkaido, Japan, and the results were compared with those obtained by the neutralization test. Furthermore, the ELISAs were applied to the epizootiological survey of wild rodents in Khabarovsk, Russia, which is an endemic area of the TBE virus.

MATERIALS AND METHODS

Cells and virus strains

BHK-21 cells were cultured in Eagle's minimum essential medium containing 8% fetal bovine serum (FBS) and were used for the neutralization tests. The 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, D-glucose and L-glutamine, and were used for the expression of the recombinant proteins.

The Oshima 5–10 strain, the Far-Eastern subtype of the TBE virus, was isolated from dogs in 1995 (21) and propagated in suckling mice inoculated intracerebrally.

Serum samples

One hundred and twenty serum samples were collected from wild rodents (24 *Apodemus speciosus*, 9 *Apodemus argenteus*, 1 *Apodemus peninsulae giliacus* and 86 *Myodes rufocanus*) that were captured in Kamiiso, Hokkaido, between August 1996 and October 1997. Thirty-five samples (10 *Apodemus speciosus* and 25 *Myodes rufocanus*) were positive for the neutralizing antibody against the TBE virus and the other 85 samples were negative. Theses samples were used to define cut-off values for the ELISAs.

Between August and September 2002, twenty-nine serum samples of wild rodents were collected in Khabarovsk, Russia, where the TBE virus is endemic, and used to evaluate the ELISAs for epidemiological research.

All serum samples were heat-inactivated at 56°C for 30 min and stored at −30°C.

Neutralization test

These tests were carried out as described previously (22). Serum samples that produced a 50% reduction in focus formation of the Oshima 5–10 strain of the TBE virus on BHK cells in 96-well plates were determined by immunohistochemical staining. Serum samples ≥1:40 were judged to be positive for neutralizing antibodies against the TBE virus.

Preparation of recombinant antigens

(1) *E. coli*-expressed antigen (EdIII)
Domain III (575aa–679aa) of the E protein of the Oshima 5–10 strain was expressed by the pET-43 vector system (Merck, Darmstadt, Germany), as a fused protein with a NusA -Tag protein (EdIII). The coding region for domain III of the E protein gene was amplified by PCR using primers: forward primer, 5′-aggCCCGGG aaaacttaagatgaaaggtct-3′; and reverse primer, 5′-cccAAGCTTa gctactccc tttttggaacc-3′. PcTBECME plasmid encoding C, prM and E proteins of Oshima 5–10 were used as a template (23). The PCR products were digested with *Sma* I and *Hind* III (Takara, Otsu, Japan), and cloned into the pET-43.1 vector. The *E. coli* Origami (DE3) pLysS (Merck) transformed with plasmid was used to express the recombinant protein. Expressed soluble EdIII was

affinity-purified in nickel-charged columns under conventional conditions according to the manufacturer's instructions (Merck). As a control, the histidine-tagged NusA protein was expressed in an *E. coli* strain carrying the intact pET-43 vector and purified under similar conditions.

(2) Subviral particles
Subviral particles were prepared as described previously (15). Briefly, a plasmid encoding the prM and E proteins of the Oshima 5–10 strain (pCAGprME) was transfected into 293T cells. At 48 hr post-transfection, the culture medium was harvested and centrifuged at 12 000 rpm for 30 min. The SPs were precipitated using polyethylene glycol (PEG) solution (10% PEG 8000, 1.9% NaCl) and were resuspended in a carbonate–bicarbonate buffer (Sigma Chemical, St Louis, MO, USA) to yield 1% of the original culture medium volume. The negative antigen was prepared from the culture medium of non-transfected 293T cells.

SDS-PAGE and Western blot

Each antigen mixed with an equal volume of lysis buffer (0.1 M Tris-HCl (pH 6.8), 4% SDS, 8% glycerol, 0.01 bromophenol blue) was heated at 90°C for 2 min and electrophoresed through 10% polyacrylamide-SDS gels. The protein bands on the gels after SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membranes (Immunobilon PVDF; Millipore, Billerica, CA, USA), then incubated with blocking buffer (Block Ace; Dai-Nippon, Osaka, Japan) and reacted for 1 hr with anti-Langat virus mouse immune ascite fluid, which is cross-reactive to the TBE virus-E proteins (1:100). After washing, the membranes were reacted with alkaline phosphatase (ALP)-conjugated antibody to mouse immunoglobulin G (IgG) (1:5000; Jackson Immuno Research, West Grove, PA, USA) for 1 hr at 37°C and washed. Protein bands were visualized by the AP Detection reagent kit (Merck) according to the manufacturer's instruction.

EdIII-ELISA

EdIII was coated onto 96-well microplates (50 μL/well, 2 μg/mL in carbonate buffer) and incubated overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBST), a blocking solution (Block Ace diluted 1:4 in DDW) was applied and incubated. The plates were washed before adding the serum samples in duplicate (50 μL/well, 1:100 dilution in PBST containing 0.5% bovine serum albumin; ELISA buffer) and incubated. Bound IgG antibodies were detected by adding 50 μL/well of peroxidase-conjugated anti-mouse IgG (1:2000 in ELISA buffer) and incubated at 37°C for 1 hr. The color reaction was developed by adding 100 μL/well of *o*-phenylenediamine dihydrochloride (Sigma, St Louis, MO, USA) in the presence of 0.07% H_2O_2 for 30 min at room temperature, and the absorbance at 450–620 nm was measured. The results for each serum sample were reported as the positive–negative difference (P–N), that is, the difference of the optical density (OD) with the positive antigen to the OD with the negative antigen; NusA -Tag protein was expressed from *E. coli*.

SP-ELISA

Rabbit anti-TBE virus E protein IgG (23) was coated onto 96-well microplates (50 μL/well, 5 μg/mL in carbonate buffer). After overnight incubation at 4°C, the plates were washed five times with PBST. A blocking solution was applied (200 μL/well) and the plates were incubated at 37°C for 1 hr. The plates were washed before adding the SP antigen (50 μL/well, 1:150 dilution in ELISA buffer) and incubated at 37°C for 1 hr. After washing with PBST, the serum samples were added in duplicate (50 μL/well, 1:100 dilution in ELISA buffer) and incubated at 37°C for 1 hr. Bound IgG antibodies were detected by adding 50 μL/well of ALP-conjugated anti-mouse IgG (1:5000 in ELISA buffer) and incubating at 37°C for 1 hr. The color reaction was developed by adding 100 μL/well of p-nitrophenyl phosphate and incubating at 37°C for 60 min, and the absorbance at 405–620 nm was measured. The results for each serum sample were reported as the P–N, that is, the difference of the OD with the positive antigen to the OD with the negative antigen, which was prepared from the supernatant of non-transfected 293T cells.

Cut-off values of each ELISA

The OD values of each ELISA were compared with the results of the neutralization test. The sensitivity and the specificity of the ELISA were calculated corresponding to each cut-off value. The sensitivity was the ratio of the number of positive sera for ELISA and the neutralization test to the number of positive sera for the neutralization test. The specificity was the ratio of the number of negative sera for ELISA and the neutralization test to the number of negative sera for the neutralization test. The cut-off value that showed the minimum difference between the sensitivity and the specificity was used as the cut-off value of each ELISA.

Results

EdIII-ELISA

To prepare the recombinant antigen, we first attempted to express the whole E proteins of the TBE virus in *E. coli*,

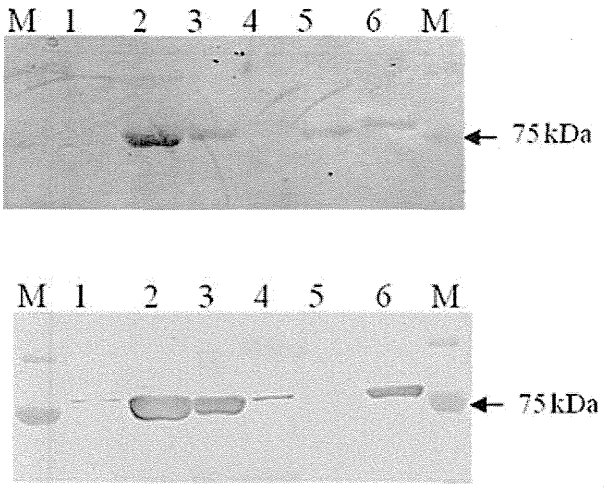

Fig. 1. Characterization of domain III of the E protein expressed as a fused protein with NusA -Tag protein (EdIII). EdIII (molecular mass, 80 kDa) was expressed in the soluble fraction of transformed *Escherichia coli* and detected by antibodies that detect tick-borne flavivirus E proteins by Western blot. Line 1: whole lysate of *E. coli* before isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Line 2: whole lysate of IPTG-induced *E. coli*. Line 3: soluble fraction of IPTG-induced *E. coli*. Line 4: insoluble fraction of IPTG-induced *E. coli*. Line 5: purified NusA -Tag protein. Line 6: purified EdIII.

but the proteins were expressed as insoluble proteins and could not be applied to the ELISA (data not shown). Next, domain III of the E protein of the Oshima 5–10 strain was expressed as a fused protein with NusA -Tag protein (EdIII). To confirm and characterize the EdIII antigen, expressed proteins were analyzed by SDS-PAGE and Western blot (Fig. 1). The EdIII (molecular mass, 80 kDa) was expressed in a soluble fraction. The expressed EdIII, not the NusA -Tag protein, was detected by antibodies that detect the E proteins of the tick-borne flavivirus by Western blot. These results indicated that EdIII can be useful as the antigen in the diagnosis ELISA.

One hundred and twenty serum samples from wild rodents captured in Kamiiso, Hokkaido, were tested for TBE virus-specific antibodies by EdIII-ELISA, SP-ELISA and the neutralization test. The detection accuracy of each ELISA was evaluated by comparing the results between the neutralization test and the ELISAs.

Figure 2 shows the sensitivity and specificity of the EdIII-ELISA by comparison with the neutralization test, using the corresponding cut-off values. The sensitivity of the EdIII-ELISA decreased with increasing cut-off values, while the specificity increased. The difference between the sensitivity and specificity was a minimum value when a cut-off value of 0.61 was used. Then at a cut-off value of 0.64, a higher specificity (80.0%, 68/85) and equal sensitivity (77.1%, 27/35) were obtained, compared to the cut-off value of 0.61 (Table 1).

SP-ELISA

The SPs were expressed by the transfection of the plasmid pCAGprME into 293T cells and precipitated using PEG solution as described previously (15). Anti-E protein rabbit IgG was prepared by immunization of a rabbit with the EdIII in order to use it as the capture antibody in the SP-ELISA (23). The anti-E protein rabbit IgG was confirmed to be reactive to both the E protein from the authentic TBE virus antigen and the SPs (Fig. 3). These results indicated that the anti-E protein rabbit IgG can be useful for the capture antibody of the diagnostic SP-ELISA.

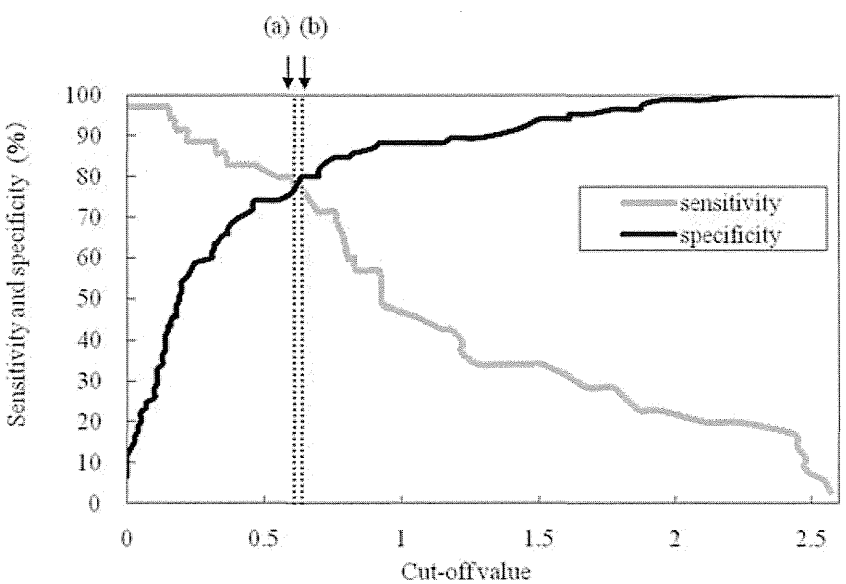

Fig. 2. Relationship between the cut-off value, sensitivity and specificity for the EdIII-ELISA. When the cut-off value was (a) 0.61, the difference between the sensitivity and specificity was at a minimum. On the other hand, when the cut-off value was (b) 0.64, the sensitivity was equal, but the specificity was higher than at a cut-off value of 0.61.

Table 1. Comparison of the results obtained by neutralization test and domain III of the E protein (EdIII)-ELISA

Neutralization test	EdIII-ELISA (cut-off value = 0.64) Positive	Negative	Total
Positive	27	8	35
Negative	17	68	85
Total	44	76	120

Figure 4 shows the sensitivity and specificity of the SP-ELISA by comparison with the neutralization test, using the corresponding cut-off values. The sensitivity of the SP-ELISA decreased with increasing cut-off values, while the specificity increased. The difference between the sensitivity and specificity was at a minimum value when a cut-off value of 0.042 was used. Then at a cut-off value of 0.089, a higher specificity (100%, 85/85) and equal sensitivity (91.4%, 32/35) were obtained, compared to the cut-off value of 0.042 (Table 2).

Application of EdIII-ELISA and SP-ELISA to the epizootiological survey

To investigate whether our ELISAs using recombinant antigens can be applied to the epizootiological survey, wild rodent samples were collected in Khavarovsk, Russia, an area in which many TBE patients were reported (24), and examined for anti-TBE virus antibodies by the ELISAs. Twenty-nine serum samples from wild rodents were tested by the EdIII-ELISA and the SP-ELISA, and the same three samples were diagnosed as positive by both ELISAs (Table 3). The three samples were also positive for the neutralization test and the other 25 samples, which were negative for the ELISAs, were also negative for the neutralization test. These results indicated that the EdIII-ELISA and the SP-ELISA can be applied to the epizootiological survey to know the distribution of the TBE virus.

DISCUSSION

To identify TBE virus-endemic areas, it is effective to conduct an epizootiological survey of wild rodents. The neutralizing test can be used for serological diagnosis of wild rodents, but it is time consuming and uses hazardous live viruses that require a high-level biosafety facility. It is also known that non-infected wild rodents sometime indicated low neutralization antibody titers by the neutralization test. Therefore, a diagnosis which is more convenient for the epizootiological survey of wild rodents is required. In this study, we tried to develop ELISAs using two recombinant antigens in the serological diagnosis of rodents for the first time.

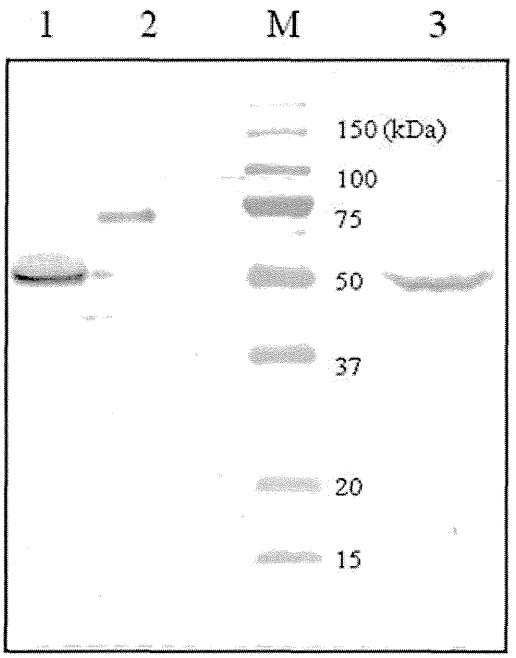

Fig. 3. Western blot analysis with anti-E protein rabbit immunoglobulin G (IgG). Each culture medium was precipitated using polyethylene glycol solution and subjected to Western blot analysis with anti-E protein rabbit IgG. The anti-E protein rabbit IgG was confirmed to be reactive to both the E protein from authentic TBE-virus antigen and subviral particles (molecular mass, 53 kDa). Lane 1: 293T cells transfected with pCAGprME (expressing subviral particles). Lane 2: non-transfected 293T cells. Lane 3: BHK cells infected with a wild TBE virus.

Domain III of the E protein was known to have the neutralizing epitopes (11) and was used for the serological diagnosis in several flaviviruses (13, 14). In this study, the recombinant domain III of the E protein was applied to the diagnosis ELISA for wild rodents. The EdIII-ELISA was shown to have a relatively high sensitivity (27/35, 77.1%) and specificity (68/85, 80.0%) as compared with the neutralization test when the cut-off value for the ELISA was set at 0.64 (Fig. 2). Eight of 35 neutralization test-positive samples were negative in the EdIII-ELISA (Table 1). Several false-positive samples showed high reactivity to the negative control antigens, NusA (data not shown). In another study it was reported that a neutralizing response to West Nile virus in naturally infected horses was induced with epitopes within not only EdIII, but also other domains (25). It was suggested that these false-negatives were due to the lack of other domains and the conformational structure of the EdIII expressed in *E. coli*, and to the presence of antibodies that have high reactivity to NusA -Tag protein.

In the flavivirus, co-expression of prM and E proteins in mammalian cells leads to the secretion of SPs to culture medium (19, 26, 27). The SPs have no viral genome

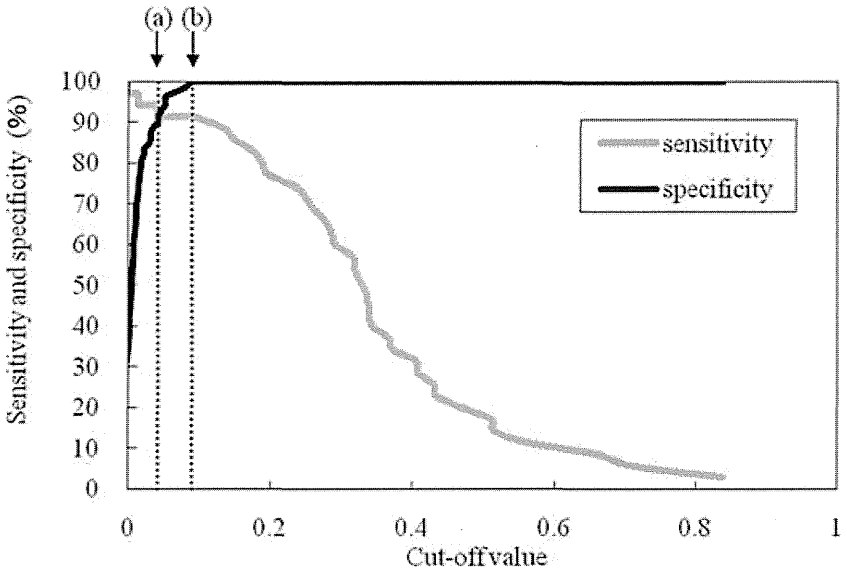

Fig. 4. Relationship between cut-off value, sensitivity and specificity for the SP-ELISA. When the cut-off value was (a) 0.042, the difference between the sensitivity and specificity was a minimum value. On the other hand, when the cut-off value was (b) 0.089, the sensitivity was equal, but the specificity was higher than at a cut-off value of 0.042.

and do not produce progeny virus, and they have similar antigenicity and immunogenicity to the native virus. Therefore, SPs have been developed as a safe and useful alternative for live viruses as the antigen for serological diagnosis tests and vaccines (18, 20, 28, 29). In this study, the SPs were used as the antigens in ELISA to detect TBE virus-infected rodents. The SP-ELISA was shown to have a very high sensitivity (32/35, 91.4%) and specificity (85/85, 100%) as compared with the neutralization test when the cut-off value for the ELISA was set at a 0.089 (Fig. 4).

In a recent study, it was reported that the antigenic structures of E proteins were disturbed when the ELISA plate was coated directly with the viral particles as solid-phase antigens (30). To avoid this, our SP-ELISA uses capture antibodies to coat the SP-antigen on the plate. And unlike infectious virions, the SPs do not require formalin inactivation, which affects the reactivity of several epitopes of the E proteins (31). In addition to the similar antigenicity between the SPs and the authentic virions, these procedural elements lead to a higher detection accuracy than the EdIII-ELISA.

In our ELISAs, anti-mouse IgG antibodies were used as the secondary antibodies. It was reported previously that anti-mouse IgG antibodies react to the IgG of various species of rodent, including *Apodemus spp.* and *Myodes spp.*, which are the main natural mammalian hosts for the TBE virus (32). The reactivity to the IgG of *Myodes rufocanus* is relatively low when compared to that to the IgG of *Mus musculus* (35.9%). The three false-negative samples in SP-ELISA were from *M. rufocanus*. It is possible that the lower reactivity might cause the false-negative results in the samples of *M. rufocanus*; however, because the most of the positive samples of *M. rufocanus* were detected, including the samples from the field survey, in a TBE virus-endemic area, the anti-mouse IgG antibodies in our ELISA are useful in large-scale epizootiological survey in various species of wild rodents.

Table 2. Comparison of the results obtained by the neutralization test and subviral particle (SP)-ELISA

Neutralization test	SP-ELISA (cut-off value = 0.089) Positive	Negative	Total
Positive	32	3	35
Negative	0	85	85
Total	32	88	120

Table 3. Results of the seroepidemiological survey by domain III of the E protein (EdIII)-ELISA, subviral particle (SP)-ELISA and the neutralization test

Species	EdIII-ELISA†	SP-ELISA‡	Neutralization test§
Myodes rufocanus	3/22	3/22	3/22¶
Apodemus agrarius	0/7	0/7	0/7
Total	3/29	3/29	3/29

†When the positive–negative difference (P–N) was >0.64, it judged to be positive for antibodies against the tick-borne encephalitis (TBE) virus. ‡P–N > 0.089 were judged to be positive for antibodies against the TBE virus. §Neutralizing titers > 1:40 were judged to be positive for neutralizing antibodies against the TBE virus. ¶The three positives that were identified by EdIII-ELISA, SP-ELISA and the neutralization test were the same samples.

The EdIII-ELISA and SP-ELISA were applied to the epizootiological survey of wild rodents in Khavarovsk, Russia, in which many TBE patients are reported annually (24). Both ELISAs could detect TBE virus-infected rodents, which were also confirmed by the neutralization test. Therefore, the ELISAs are suitable for screening to detect TBE virus-infected rodents by investigating a number of rodent samples, and they are useful for specifying a TBE virus-endemic area.

In summary, we developed the ELISAs using domain III of the E proteins and the SPs as the antigens. The ELISAs had high sensitivity and specificity, and it was shown that SP antigens had higher detection accuracy than domain III antigens. The ELISAs were also shown to be applied to the epizootiological research in TBE virus-endemic area. This is the first study to show the serological diagnosis of wild rodents using recombinant antigens and the ELISAs can be safe and useful in the detection of TBE virus-infected wild rodents in epizootiological research.

ACKNOWLEDGMENT

This work was supported by Grants-in-Aid for Scientific Research (22780268) and the global COE program from the Ministry of Education, Science, Sports and Culture of Japan, and Health Sciences Grants for Research on Emerging and Re-emerging Infectious Disease from the Ministry of Health, Labor and Welfare of Japan.

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