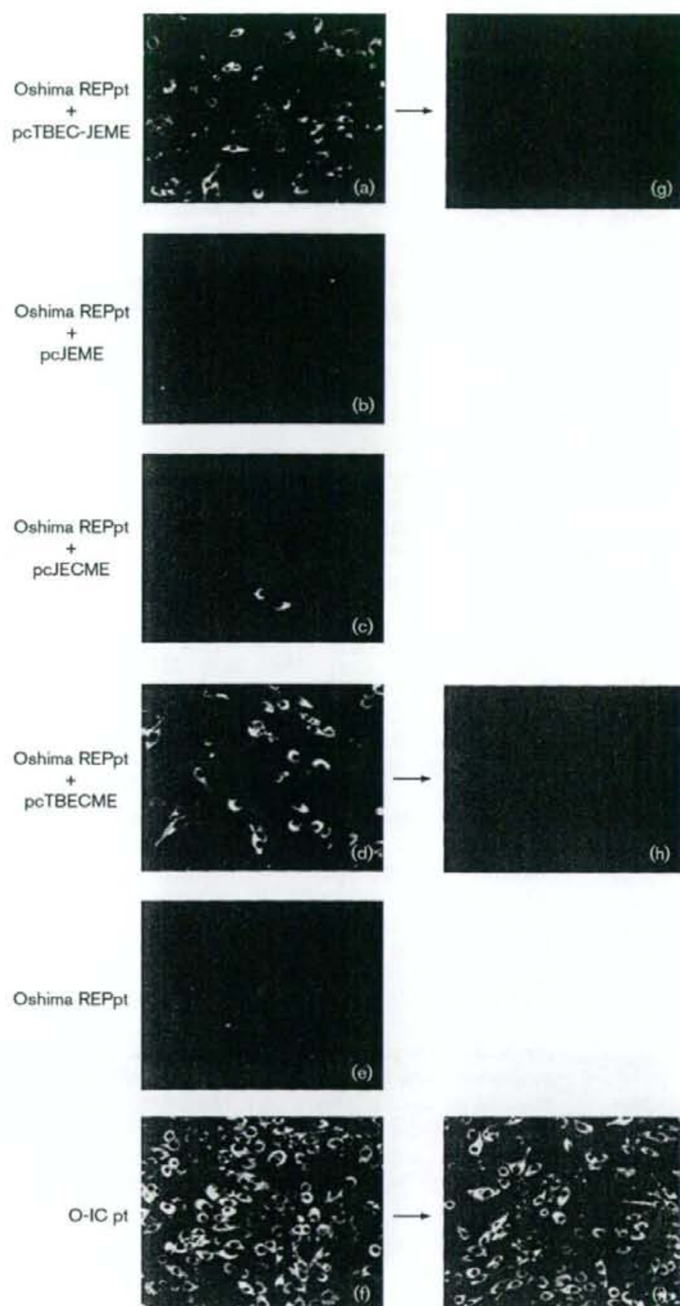

Fig. 2. Secretion of chimeric particles with single-round infectivity. (a–f) Fresh BHK-21 cells were infected with the culture supernatants of cells that were transfected with *in vitro*-synthesized Oshima REPpt RNA and (a) pcTBEC-JEME, (b) pcJEME, (c) pcJECME or (d) pcTBECME, or (e) Oshima REPpt alone. As a control (f), RNA from the full-length TBE virus infectious cDNA O-IC pt was used. (g–i) Reinfection of fresh BHK-21 cells with the culture supernatants: (a)→(g), (d)→(h) and (f)→(i). Expression of viral proteins was visualized by immunofluorescence using anti-NS3 antibodies.

E-specific antibodies for the JE virus prM and E protein bands in Oshima REPpt replicon-electroporated cells that were transfected sequentially with the pcTBEC-JEME, pcJECME and pcJEME plasmids. It has been reported previously that the C–prM junction is cleaved first by the NS3 protein on the cytoplasmic side, and then cleaved continuously by the ER signal peptidase on the ER-luminal side (Amberg *et al.*, 1994; Lobigs, 1993; Sato *et al.*, 1993).

In this case, prM protein bands were detected as independent forms from the C proteins and no C–prM polyprotein band was detected, which indicates that the C–prM junctions were cleaved by viral NS3 protease derived from the expression of the TBE virus replicon RNA. On the other hand, viral protein secretion was observed in the culture supernatants of replicon-electroporated cells that were transfected sequentially with the pcTBECME, pcTBEC-JEME and pcJEME plasmids. However, low levels of E protein were secreted from cells that were transfected with Oshima REPpt RNA and pcJECME. Furthermore, the harvested supernatant was used to infect BHK-21 cells for titration of infectious VLPs (Fig. 3b). A relatively high titre of infectious VLPs ($>10^6$ IU ml^{-1}) was secreted from cells that were transfected with both the pcTBECME and pcTBEC-JEME plasmids, whereas few VLPs (250 IU ml^{-1}) were secreted from cells that were transfected with Oshima REPpt RNA and pcJECME. Semiquantitative RT-PCR showed that the secretion levels of replicon RNAs also corresponded to the infectious titre of VLPs from cells transfected with each plasmid (Fig. 3c). On the other hand, pcJEME plasmid transfection into replicon-electroporated cells resulted in no secretion of infectious VLPs, despite the secretion of viral structural proteins. This viral protein secretion was due to the secretion of subviral particles (SPs), which consist of a viral envelope without nucleocapsid or genomic RNA, as reported in our previous study (Konishi *et al.*, 2001). These data indicate that the efficient packaging and incorporation of nucleocapsid require homologous interactions between flavivirus C proteins and non-structural proteins or genomic RNA, but not between C proteins and viral envelope proteins.

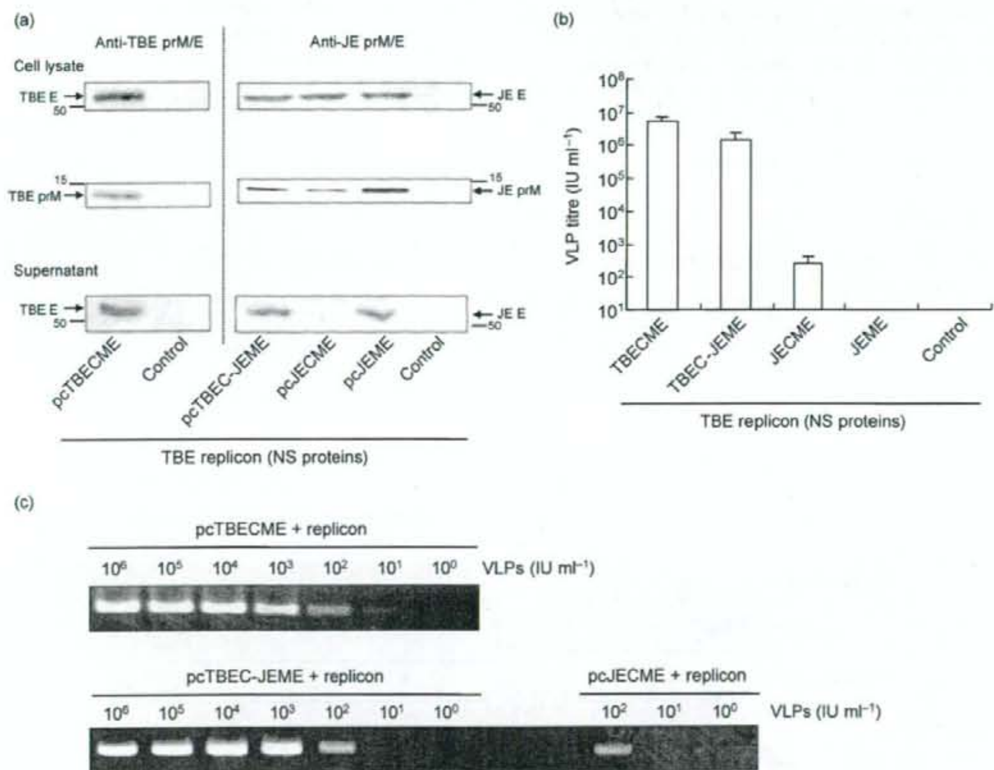

Fig. 3. Kinetics of VLP secretion. (a) Detection of flavivirus structural proteins. TBE virus replicon-electroporated BHK-21 cells were transfected sequentially with pcTBECME, pcTBEC-JEME, pcJECME, pcJEME or control plasmid. At 36 h post-transfection of the plasmid, cell lysate and supernatant samples were separated by SDS-PAGE under non-reducing conditions and subjected to Western blotting. Viral proteins were visualized by specific antibodies against the TBE virus prM/E (left panel) and JE virus prM/E (right panel) proteins. Positions of individual proteins are marked; molecular mass (in kDa) is indicated. (b) Titres (IU) contained in the culture fluids were determined by infectivity assay to BHK-21 cells. The data are means from four independent experiments (error bars indicate SEM). (c) Detection of replicon RNAs packaged in VLPs. Secreted VLPs were diluted serially and subjected to RT-PCR for the detection of TBE virus replicon RNA.

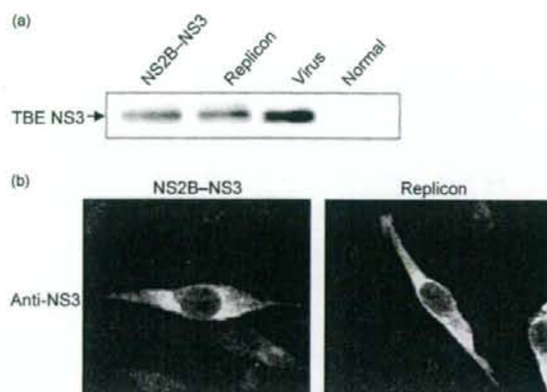

Fig. 4. (a) Expression of NS3 proteins in BHK-21 cells transfected with pcTBENS2B/3 plasmid and replicon RNAs. TBE virus-infected cells and untreated cells were used as a control. (b) Localization of NS3 proteins in BHK-21 cells transfected with pcTBENS2B/3 plasmid (left panel) and replicon RNAs (right panel). Expression of NS3 proteins was visualized by anti-NS3 antibodies at 36 h post-transfection.

Secretion of subviral particles from cells that express chimeric structural proteins and NS2B–NS3 proteins

It is known that expression of the flavivirus envelope proteins prM and E leads to budding and secretion of SPs. To examine the mechanism involved in the differential secretion of infectious VLPs observed for pcTBEC-JEME and pcJECME transfection of replicon-electroporated cells, we investigated SP secretion from cells that expressed C–prM–E polyproteins with TBE virus NS2B–NS3 protease. The pcTBENS2B/3 plasmid, which expresses TBE virus NS2B–NS3 polyproteins, was prepared. The same amount of NS3 was expressed and similar cytoplasmic localization was observed in cells transfected with pcTBENS2B/3 compared with those transfected with the TBE virus replicon RNAs (Fig. 4).

Individual plasmids that express flavivirus C/prM/E structural proteins (pcTBECME, pcTBEC-JEME or pcJECME) were transfected alone or together with pcTBENS2B/3 into BHK-21 cells. When the C/prM/E viral structural proteins were expressed without NS2B–NS3 proteins, C–prM polyprotein bands larger than those seen for prM were detected by the prM-specific antibodies (Fig. 5; lower panel with cell lysate samples). When the

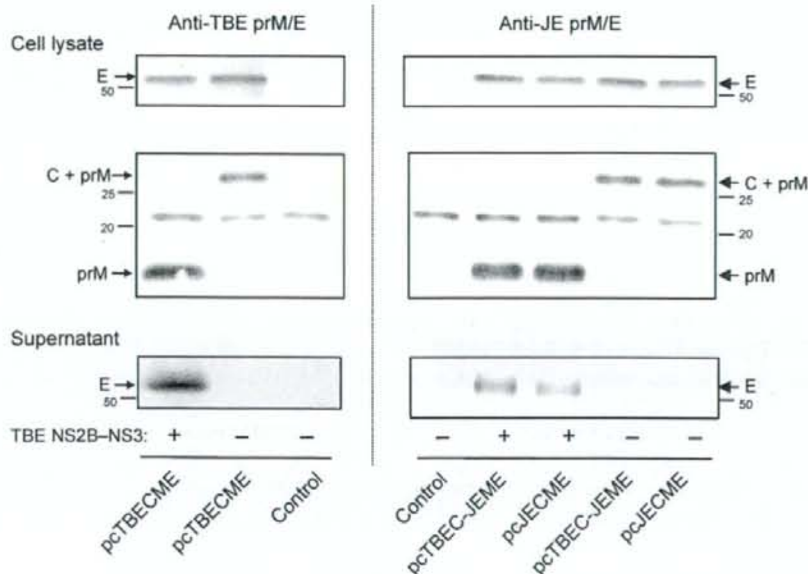

Fig. 5. Detection of flavivirus structural proteins in transfected BHK-21 cells. BHK-21 cells that were transfected with (+) or without (−) the pcTBENS2B/3 plasmid were co-transfected with pcTBECME, pcTBEC-JEME, pcJECME or control plasmid. At 36 h post-transfection, cell lysate and supernatant samples were separated by SDS-PAGE under non-reducing conditions and subjected to Western blotting. Viral proteins were visualized by using specific antibodies against the TBE virus prM/E (left panel) and JE virus prM/E (right panel) proteins. Positions of individual proteins are marked; molecular mass (in kDa) is indicated.

viral structural proteins were expressed with TBE virus NS2B–NS3 proteins, the prM protein bands were detected as separate forms from the C proteins and no C–prM polyprotein band was detected, which indicates that the C–prM junctions were cleaved by TBE virus NS3 protease, as observed for the co-expression of the TBE virus replicon RNA (Fig. 3). On the other hand, whilst no viral protein was secreted without NS2B–NS3 expression, viral protein secretion was observed in all supernatant samples of cells that expressed the flavivirus C/prM/E structural proteins together with TBE virus NS2B–NS3 proteins (Fig. 5; panel with supernatant samples), indicating that SPs are secreted by cleavage of the C–prM junction. This suggests that the low level of VLP secretion from cells that were transfected with pcJECME and TBE virus replicon RNA (Fig. 3) is not due to the budding property of the viral envelope prM/E proteins after processing by NS2B–NS3 protease, and that other factors, such as the mechanism for genome replication and packaging, are involved in the efficient secretion of infectious chimeric VLPs.

Neutralizing test for VLP infection of BHK-21 cells

To confirm the antigenic characteristics of the secreted VLPs, the reactivities of VLPs with anti-flavivirus antibodies were examined. The neutralization test for VLP infection of BHK-21 cells was performed by using the mouse mAbs and polyclonal antibodies against TBE virus, Langat virus (tick-borne flavivirus) and JE virus. As shown in Table 1, mAb 1H4, anti-TBE virus polyclonal antibodies and anti-Langat virus polyclonal antibodies neutralized TBE-envelope VLP infectivity at almost the same concentrations as they did native TBE virus infectivity. In addition, the anti-JE virus polyclonal antibodies neutralized JE-envelope VLP infectivity at almost the same concentration as they did JE virus infectivity. The cross-reactive mAb 4H8 showed similar reactivities for the VLPs and naïve viruses. These data show that the envelope glycoproteins of VLPs have the same antigenicity as those of authentic virus particles.

Table 1. Neutralizing titres of antibodies against infectious particles of flaviviruses

Data are reciprocal numbers of the highest serum dilution that reduced the virus focal count by 50 %.

Antibody	VLPs*			
	TBE-envelope	JE-envelope	TBE virus	JE virus
mAbs†				
1H4	>640	<20	>640	<20
4H8	40	80	40	80
Polyclonal antibodies				
Anti-TBE virus	160	<20	320	<20
Anti-Langat virus	>640	<20	>640	<20
Anti-JE virus	<20	320	<20	640

*VLPs were prepared from BHK-21 cells transfected with Oshima REPpt replicon and pcTBEC-JEME (TBE-envelope VLPs) or pcJECME (JE-envelope VLPs).

†1H4, Specific for tick-borne flavivirus; 4H8, cross-reactive against tick-borne and mosquito-borne flavivirus.

Infectivities of TBE-envelope and JE-envelope VLPs for arthropod cells

The JE-envelope VLPs contain envelope proteins that are derived from the mosquito-borne JE virus and carry nucleocapsid and replicon RNA derived from the tick-borne TBE virus. Therefore, we examined the susceptibilities of cell lines derived from arthropods to infection with chimeric JE-envelope VLPs. ISE6 cells, derived from *I. scapularis*, and C6/36 cells, derived from *A. albopictus*, were infected with the TBE-envelope VLPs, JE-envelope VLPs, TBE virus or JE virus, and viral protein production was detected by IFA.

As shown in Fig. 6, the tick cell line ISE6 was susceptible to infection by the TBE virus and TBE-envelope VLPs and the viral proteins showed a cytoplasmic distribution. However, the ISE6 cells were not susceptible to infection by the JE virus or JE-envelope VLPs, although the JE-envelope VLPs contained replicon RNA derived from the TBE virus. On the other hand, the mosquito cell line C6/36 showed no signs of infection by the TBE virus or TBE-envelope VLPs. The C6/36 cells were positive for viral antigen after infection with the mosquito-borne JE virus. Interestingly, the C6/36 cells that were infected with JE-envelope VLPs were not positive by IFA.

In order to reveal the mechanism involved in the lack of susceptibility of tick and mosquito cells to JE-envelope VLPs, the internalization of VLPs was examined (Fig. 7). Following 1 h VLP absorption, replicon RNA was detected in ISE6 cells that were infected with TBE-envelope VLPs, whereas most of the JE-envelope VLPs remained in the supernatant and no replicon RNA was detected intracellularly. This indicates that the entry of JE-envelope VLPs into ISE6 cells is inefficient and, thus, no viral proteins are detected by IFA (Fig. 6). In contrast, the JE-envelope VLPs entered the C6/36 cells after 1 h VLP absorption, whereas the TBE-envelope VLPs remained in the supernatant (Fig. 7a). However, intracellular replicon RNAs disappeared at 8 h post-infection (Fig. 7b). Taken together with the IFA results (Fig. 6), this suggests that JE-envelope VLPs can enter C6/36 mosquito cells, but cannot replicate therein, due to fact that their genomic replicon RNA is derived from the tick-borne TBE virus. Direct transfection of C6/36 cells with TBE virus full-length RNA or replicon RNA resulted in neither viral protein production nor virus particle secretion, as reported previously (Mandl *et al.*, 1991).

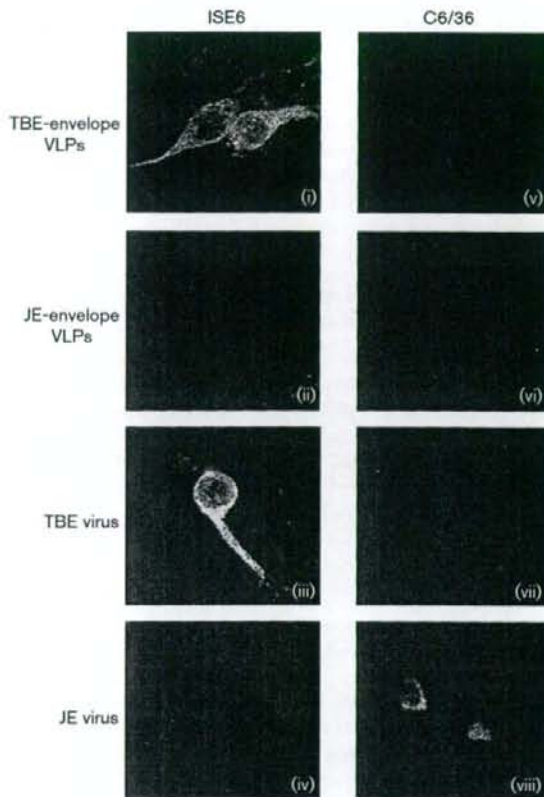

Fig. 6. Susceptibility of ISE6 (tick) and C6/36 (mosquito) cell lines to infectious flavivirus particles. ISE6 (i–iv) and C6/36 (v–viii) cells were infected with TBE-envelope VLPs (i, v), JE-envelope VLPs (ii, vi), TBE virus (iii, vii) or JE virus (iv, viii), prepared from the culture supernatants of BHK-21 cell cultures. Viral protein production was visualized by immunofluorescence using specific antibodies, as described in Methods.

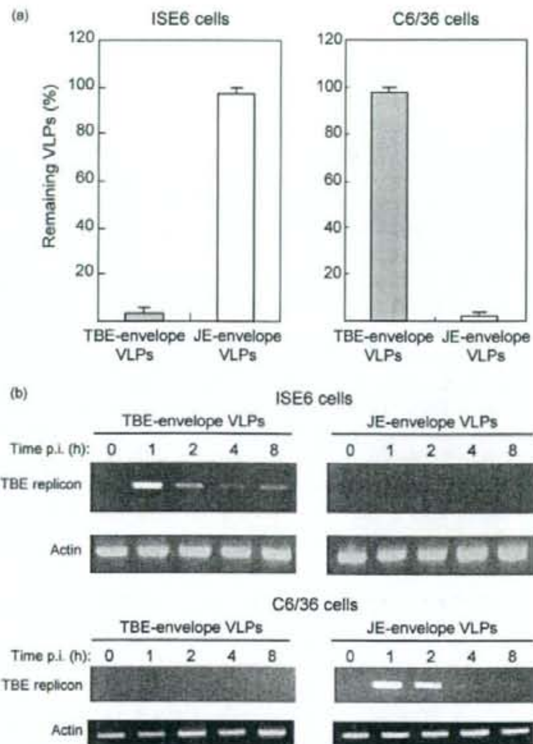

Fig. 7. (a) Internalization of TBE- or JE-envelope VLPs into arthropod cells. ISE6 and C6/36 cells were infected with VLPs. After 1 h absorption, the supernatants were harvested and remaining VLPs were titrated. Results are expressed as percentages of remaining VLPs compared with untreated controls. The data are means from three independent experiments (error bars indicate SEM). (b) Detection of replicon RNA from arthropod cells infected with TBE- or JE-envelope VLPs. ISE6 and C6/36 cells were infected with the VLPs. At each time point post-infection (p.i.), RNA samples were extracted and subjected to RT-PCR for the detection of TBE virus replicon RNA. Actin mRNA was used as an internal control.

DISCUSSION

In this study, we developed, for the first time, chimeric VLPs between mosquito-borne and tick-borne flaviviruses, using the *trans*-packaging system for TBE virus subgenomic replicons. In recent years, the development of reverse-genetic technologies has enabled the construction of flavivirus chimeras in various combinations (Caufour *et al.*, 2001; Guirakhoo *et al.*, 2000, 2001; Huang *et al.*, 2000; Mathenge *et al.*, 2004; Monath *et al.*, 1999; Pletnev & Men, 1998; Pletnev *et al.*, 1992, 2002). These chimeras may be useful tools for studying the biological features of flaviviruses and for vaccine development. However, several aspects of flavivirus chimeras require further study. The family *Flaviviridae* contains many important human pathogens that cause severe symptoms and high mortality (approx. 30 %). Thus, recent studies of flavivirus chimeras have tended to use less pathogenic or vaccine strains of flavivirus for the development of live-attenuated vaccines. On the other hand, flavivirus VLPs have the same antigenicity and virus-entry characteristics as the wild-type virus, and the single-round infectivity of VLPs allows infection experiments to be conducted under BSL-2 conditions (Gehrke *et al.*, 2003; Khromykh *et al.*, 1998; Scholle *et al.*, 2004; Yoshii *et al.*, 2005). Therefore, our strategy is that chimeric VLPs can substitute for chimeric viruses in investigations of the biological properties of flaviviruses.

Chimeric VLPs that packaged the TBE replicon were recovered only following *trans*-expression of the TBE virus

C and JE virus prM/E proteins. In many studies of flavivirus chimeras, chimeric viruses have been recovered successfully by replacement of the viral envelope protein prM and E genes by those from other flaviviruses. These data indicate that the C proteins (or nucleocapsid) interact non-specifically with viral envelope proteins and that this interaction is not important for the assembly of virus particles. On the other hand, in experiments using *trans*-expression of the JE virus C/prM/E proteins, we failed to recover chimeric VLPs. Similar results have been shown previously in several studies of chimeric viruses, in which substitution of the C/prM/E protein genes of a tick-borne flavivirus with those of mosquito-borne flaviviruses resulted in inefficient recovery of chimeric virus (Chambers *et al.*, 1999; Pletnev & Men, 1998; Pletnev *et al.*, 1992) and, in a study by Harvey *et al.* (2004), packaging efficiency of dengue type 2 virus replicon RNAs by *trans*-expression of the KUN virus C/prM/E proteins was lower than that of KUN virus replicon RNA; the detailed mechanism underlying this finding has not been analysed.

It is known that the C terminus of the C protein is processed by the activity of the viral NS2B–NS3 protease, and that the N terminus of prM is subsequently cleaved by the cellular signal peptidase (Amberg & Rice, 1999; Amberg *et al.*, 1994; Stocks & Lobigs, 1998; Yamshchikov & Compans, 1995; Yamshchikov *et al.*, 1997). The processed prM protein then forms a heterodimer with the E protein, which is essential for the maturation and assembly of infectious particles (Allison *et al.*, 1995; Konishi & Mason, 1993; Lorenz *et al.*, 2002). In cells that expressed the JE virus C/prM/E structural proteins and TBE virus replicon or NS2B–NS3, prM proteins were detected in the form cleaved from the C protein, which indicates that the JE virus C–prM junctions are cleaved by the TBE virus NS2B–NS3 protease (Figs 3 and 5). However, in spite of the processing of the C–prM junctions, the expression of JE virus C/prM/E proteins in cells that harboured the TBE virus replicon resulted in a low level of secretion of viral proteins compared with cells that expressed the TBE virus C and JE virus prM/E proteins or the TBE virus C/prM/E proteins, which secreted VLPs that packaged the replicon RNA. The difference between the *trans*-expressed polyproteins is a reflection of whether the respective C proteins are homologous to the genomic replicon RNA. Thus, in addition to the processing of C–prM junctions, other factors related to the homology between the C protein and genomic RNA regulate the assembly of VLPs that package replicon RNA.

Efficient secretion of E, in the form of non-infectious SPs, was observed by the expression of JE virus C/prM/E proteins and TBE virus NS2B–NS3 proteins, but few infectious VLPs and fewer E proteins were secreted by the expression of JE virus C/prM/E proteins in TBE replicon-transfected cells. This indicated that some factor involved in the replication of replicon RNA regulated the process of viral particle assembly and secretion and retained viral structural proteins intracellularly. Recent studies of flavivirus non-structural proteins have revealed the involvement of NS2A and NS3 in the assembly and/or release of infectious virus particles (Khromykh *et al.*, 2000; Kummerer & Rice, 2002; Liu *et al.*, 2002) and it has been reported that the packaging of flavivirus genomic RNA into infectious particles is coupled to genome replication (Khromykh *et al.*, 2001). From these studies, it has been concluded that homologous interaction between viral C and non-structural proteins or genomic RNA is important for the efficient assembly and secretion of infectious particles packaging genomic RNA. No signals or motifs in flavivirus RNA or C protein that determine the specificity of packaging have yet been defined. However, these findings can contribute to future studies of packaging signals and increase understanding of how flavivirus virions are assembled and secreted.

It has been shown that, in general, arthropod-borne flaviviruses infect either mosquito or tick cells (Lawrie *et al.*, 2004). However, it is not known whether the ability of certain flaviviruses to infect certain cells but not others is due to viral entry into the cells or to replication and subsequent release from infected cells. The chimeric VLPs developed in our study have viral envelopes that are derived from mosquito-borne JE virus, and nucleocapsid (C protein plus subgenomic replicon RNA) derived from tick-borne TBE virus. Virus particles of flaviviruses enter cells by receptor-mediated endocytosis, the viral envelope fuses with the endosomal membrane and then the genomic RNAs are uncoated from the nucleocapsid and replicate (Heinz *et al.*, 2004). Thus, our chimeric VLP system has the advantage that it can separate particle-internalization events from sequential viral-replication events. As shown in Figs 6 and 7, TBE virus and JE virus infected either ISE6 or C6/36 cells, and TBE-envelope VLPs infected only ISE6 cells. However, no TBE virus NS proteins were produced in either tick or mosquito cells that were infected with JE-envelope VLPs. RT-PCR revealed that the TBE- and JE-envelope VLPs could enter ISE6 or C6/36 cells, and that the TBE virus replicon RNAs could not replicate in C6/36 cells that were infected with JE-envelope VLPs. Similar results were reported in a study by Pletnev *et al.* (1992), in which a chimeric virus of TBE virus/dengue type 4 virus that contained prM/E protein genes from the TBE virus was restricted in its ability to enter C6/36 cells. It is not known whether JE virus genomic RNA can replicate in tick cells, but these data indicate that vector-specific factors in arthropod cells may be required for each step of virus entry and replication during flavivirus infection. Although the susceptibility of a tick- or mosquito-derived cell to a particular arbovirus does not always reflect vector association, it is a useful indicator.

In addition to their use in investigations of the biological properties of flaviviruses, chimeric VLPs can be applied to serological diagnosis as a substitute for neutralization testing, which uses infectious viruses. As shown in Table 1, infections with TBE- and JE-envelope VLPs were neutralized by mAbs and immune sera with titres similar to those

used to neutralize the TBE and JE viruses, indicating that the chimeric VLP system is an effective alternative to the use of native flaviviruses in neutralization tests. This chimeric VLP-based neutralization system does not require a high-level containment laboratory, as the subgenomic replicon RNA packaged in the VLPs does not have the genes that encode the viral structural proteins and thus is unable to produce infectious progeny viruses. Furthermore, by replacement of the prM/E genes with those from other flaviviruses in plasmids used for the *trans*-expression of viral structural proteins, the chimeric VLP system can be adjusted easily to develop neutralization tests for a variety of flaviviruses, as substitutes for tests involving the native viruses.

In summary, we have generated chimeric VLPs that comprise the JE virus envelope and TBE virus nucleocapsid, which contains the subgenomic replicon of the TBE virus. JE-envelope VLPs were secreted when the TBE virus C protein and JE virus prM/E proteins were expressed in TBE virus replicon-electroporated cells, but not when all of the structural proteins of JE virus were expressed. The TBE- and JE-envelope VLPs were neutralized by antibodies against flaviviruses with efficacies similar to those for the native TBE and JE viruses. The infectivities of the TBE- and JE-envelope VLPs for tick and mosquito cells suggest that vector host cell-specific factors are involved in each step of flavivirus entry and replication during arthropod infection.

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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′-TGTG*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 reexamined 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]

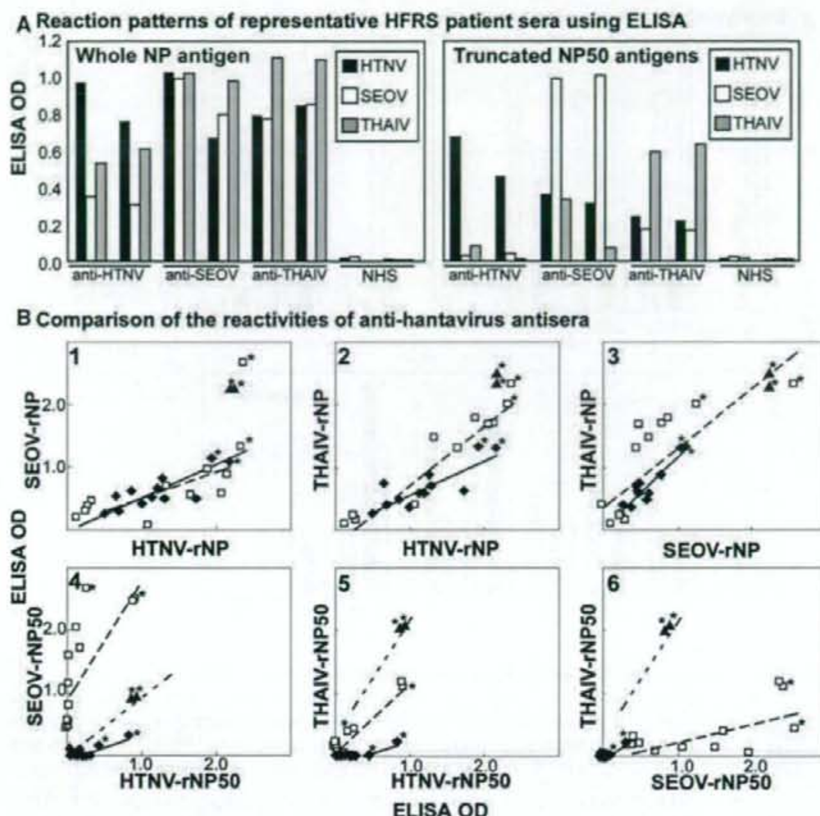

Fig. 1 Reactivities of representative and groups of patient sera against recombinant and truncated NP antigens in ELISA. **a** Reaction patterns of representative HFRS patient sera. Anti-HTNV patient sera were obtained from China. Anti-SEOV patient sera were obtained from Japan to Korea, associated with 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

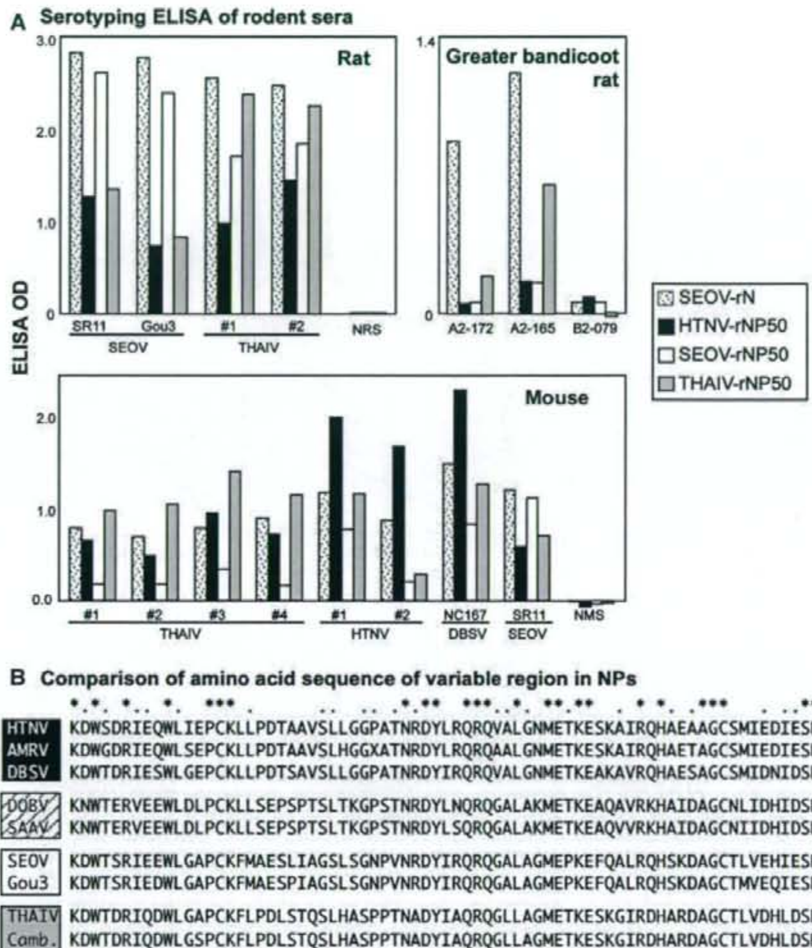

Fig. 2 Reactivities of rodent sera to truncated N antigens of HTNV, SEOV, and THAIV and comparison of the amino acid sequences of the variable region in NP. **a** Hantavirus-infected rat and mouse sera were subjected to serotyping ELISA. Sera from laboratory rats immunized with SEOV strain SR-11 or Gou3 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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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)[36] 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