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Citation	Archives of Virology, 156(11), 1931-1941 https://doi.org/10.1007/s00705-011-1066-0
Issue Date	2011-11
Doc URL	http://hdl.handle.net/2115/50373
Rights	The original publication is available at www.springerlink.com
Type	article (author version)
Additional Information	There are other files related to this item in HUSCAP. Check the above URL.
File Information	AoV156-11_1931-1941.pdf

Construction of a replicon and an infectious cDNA clone of the Sofjin strain of the far-eastern subtype of tick-borne encephalitis virus

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Abstract Tick-borne encephalitis virus (TBEV) causes severe encephalitis in humans. The Sofjin-HO strain is the prototype strain of the TBEV far-eastern subtype, and is highly pathogenic in a mouse model. In this study, we constructed replicons and infectious cDNA clones of the Sofjin-HO strain. The replication of the replicon RNA was confirmed, and infectious viruses were recovered from the infectious cDNA clone. The recombinant viruses showed similar virulence characteristics to those of the parental virus. While characterizing the replicon and infectious cDNA, several amino acid differences derived from cell culture adaptations were analysed. The amino acids differences at E position 496 and NS4A position 58 were found to affect viral replication. The Gly- or Ala-to-Glu substitution at E position 122 was shown to increase neuroinvasiveness in mice. These replicons and infectious cDNA clones are useful in revealing the viral molecular determinant involved in the replication and pathogenicity of TBEV.

148 words

Introduction

Tick-borne encephalitis virus (TBEV) belongs to the genus *Flavivirus* of the family *Flaviviridae*, and can cause fatal encephalitis in humans. TBE is endemic in Europe, Russia and Far-East Asia, and more than 10,000 cases of the disease are reported every year. TBE is a significant public health problem in these endemic regions.

TBEV can be divided into three subtypes; Far-Eastern, European and Siberian [17]. The Far-Eastern subtype, previously known as Russian spring-summer encephalitis virus, causes a severe clinical manifestation and shows a higher case fatality rate (5-20%) than the other two subtypes [32, 56]. The European subtype, also known as Central european encephalitis virus, produces biphasic febrile illness and milder encephalitis, and fatality rates are 1-2% [5, 19, 30]. The Siberian subtype causes less severe disease (case fatality rates, 2-3%) than the Far-Eastern subtype and is often associated with chronic disease [2, 39, 45, 46]. However, little is known of the mechanisms of the differing clinical manifestations among the three subtypes.

The flavivirus genome consists of a positive-polarity, single-stranded RNA of approximately 11 kb, which encodes three structural proteins, i.e. the core (C), premembrane (prM), and envelope (E) proteins, and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5), within a single long open reading frame [9], which is co-translated and cleaved post-translationally. The 5'- and 3'-UTRs have predicted secondary structures that are implicated in viral replication, translation, and packaging of the genome [16].

Previous pathological examination in a mouse model have demonstrated that the Far-Eastern subtype is highly pathogenic and causes a severe and debilitating encephalitic disease, similar to that seen in humans [11]. The Sofjin strain has been used as the prototype strain of the Far-Eastern subtype [56]. In a previous study, we showed that the Sofjin strain was more pathogenic than the Oshima strain, isolated in Japan from a mouse model [11, 13]. The amino acid identity between the two strains is more than 98%[13]. Comparison of the pathogenicity of Sofjin and Oshima strains reveals significant information about viral molecular determinants that are involved

in the differing virulence of the strains, and the pathogenicity of TBEV.

Replicon and infectious cDNA clones are useful in investigating genetic determinants of flavivirus replication and virulence. Replicon and infectious cDNA clones have been generated for multiple flaviviruses [6, 8, 16, 18, 23-25, 27, 31, 37, 42, 47, 48, 51-53, 55]. In a previous study, we constructed a replicon and a full-length infectious cDNA clone of the Far-Eastern subtype Oshima 5-10 strain [20, 21], but those of the Sofjin strain have not been constructed. The Sofjin strain has been passaged many times, which has caused the emergence of various variants. Thus, it is important to construct a cDNA clone of the Sofjin strain and to analyze the characteristics of variants in the parental virus.

In this study, we constructed and characterized replicons and infectious cDNA clones of the Sofjin strain. While characterizing the several clones of replicon and infectious cDNA, several amino acid differences derived from cell culture adaptations were shown to affect viral replication and virulence in mice.

Materials and Methods

Cells and virus

Baby hamster kidney (BHK) cells were grown in Eagle's minimal essential medium (MEM), supplemented with 8% fetal calf serum (FCS). The Sofjin-HO strain (Accession No. 062064) was first isolated from the brain of a TBE patient in Khabarovsk in 1937 [56]. The virus (unknown passage history) was kindly given by Dr. Ohya (National Institute of Infectious Diseases, Tokyo, Japan) in 1967, and further passaged 7 times in suckling mouse brain and 2 times in BHK cells. The recombinant viruses, Oshima-IC and Sofjin-IC were prepared from the full-length infectious cDNA clone.

Plasmid preparation

Sofjin-REP plasmid

Total cellular RNA was extracted from BHK cells infected with the Sofjin strain using Isogen (Nippon Gene). Viral RNA was reverse-transcribed with random primer using Superscript II reverse transcriptase (Invitrogen). Three fragments of Sofjin cDNA were amplified using Platinum Taq polymerase (Invitrogen; Fig.1). Primers were designed on the basis of the nucleotide sequence of Sofjin strain (AB062064). The first fragment (nt 1-239) had an SP6 promoter recognition site that preceded the first base of the viral genome. The first fragment was designed to fuse the fragment of C in-frame to a C-terminal fragment of E, which served as a signal sequence for NS1. A synthetic oligonucleotide was used to add an *Avr*II site at nt 239-244 and nt 2291–2296. This silent mutation was engineered to permit ligation of the second fragment (nt 2292-6335) and the proper translation of the signal sequence for NS1. The last fragment (nt 6336–10894) had an *Spe*I restriction endonuclease site. The fragments were resolved by gel electrophoresis and purified by standard methods (Qiagen kits). The fragments were digested with restriction endonucleases and ligated into the low-copy plasmid pGGV$_{S209}$[16] (Fig.1).

Sofjin-IC plasmid

The full-length infectious cDNA clone, Sofjin-IC, was created by the insertion of the coding regions for C, prM and E into Sofjin-REP plasmid. C, prM, and E fragments (nt 239–2292) were amplified by RT-PCR from the parent Sofjin-HO virus RNA. The sense primer included a *Spe*I restriction endonuclease site. The PCR products were digested with *Spe*I and *Avr*II and inserted into the Sofjin-REP plasmid, which was predigested with *Avr*II. To construct the Sofjin-REP or Sofjin-IC plasmid with amino acid differences, the fragments with the each amino acid were amplified by RT-PCR and subsequently cloned into pCR2.1 plasmid (TA cloning kit; Invitrogen). These intermediate plasmids were cut with the restriction enzymes described above and used to substitute the regions that contained the substitutions (Fig. 2).

Oshima-IC and Oshima-REP

Oshima-IC, which encoded the full-length cDNA of TBEV Oshima strain, was prepared as described previously [20]. The Oshima-REP plasmid was used for the preparation of replicon RNAs of the Oshima strain, as described previously[21].

mRNA transcription and electroporation

Replicon and infectious clone plasmids were linearized with *Spe*I and transcribed into the RNA using mMESSAGE mMACHINE SP6 Kit (Ambion), as described previously [15]. The mRNA samples were treated with DNase I and precipitated with LiCl. The precipitated RNA was dissolved in 30 µL DEPC-treated water. mRNA was transfected into BHK cells using the trans IT-mRNA Transfection Kit (mirus) or electroporation as described previously[20].

Immunofluorescence assay

Cells in 8-well chamber slides were subjected to 4% paraformaldehyde fixation for 30 min, rinsed in PBS and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 4 min. Cells were blocked with 2% BSA in PBS and then reacted with mouse TBEV hyperimmune ascites fluid. After washing with PBS, the cells were labeled with mouse secondary antibodies conjugated with Alexa 555. The cells were examined with a fluorescence microscope.

RNA extraction, reverse transcription, and TaqMan assay

Replicon RNA was extracted using Isogen (Nippon Gene) according to the manufacturer's protocol. The RNA samples were treated with DNase I and precipitated with LiCl. Total RNA was quantitated by measuring the OD_{280} value. First-strand cDNA was synthesized using Super Script II reverse transcriptase (Invitrogen) as follows: 0.4 µg total RNA was mixed with 2.5 µg random primer (Invitrogen) and the mixture was incubated at 70°C for 10 min and at 25°C for 10 min. After incubation, 2 µL 5× First Strand Buffer, 0.5 µL 10 mM dNTP mix, 1µL DDT, and 0.5 µL Super Script II reverse transcriptase were added. The reaction mixture was sequentially incubated at 42°C

for 50 min and then 70°C for 15 min. The synthesized cDNA was stored at -80°C.

The TaqMan assay was performed with the TaqMan universal PCR Master Mix (Applied Biosystems) as follows: 2.5 µL cDNA was mixed with 900 nmol each of the forward and reverse primers, 200 nmol multi-probe, and 12.5 µL TaqMan PCR universal Master Mix, and DEPC-treated water (Nippon Gene) was added to give a final volume of 25 µL. Primers and multi-probe were prepared, as described in Schwaiger's study[44]. Real-time PCR was performed using an ABI Prism 7000 (Applied Biosystems) with the following conditions: 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C, and 1 min at 60°C. The TaqMan assay was performed in duplicate for each sample and a water control was included in each assay. Samples with a cycle threshold (C_t) value < 40 and a change in the magnitude of the fluorescent signal (ΔR_n) > 0.5 were considered positive. To calculate the concentration of the replicon RNAs, the Oshima-REP plasmid DNA was used as the standard, as described previously [54].

Virus titration

Plaque assays were carried out with BHK cells using 12-well plates. Serial 10-fold dilutions of organ suspensions or culture medium from infected cells (100 µL) were inoculated with the cells and they were incubated for 1 h at 37°C before 1.5% CMC-MEM (1 mL/well) was added. Incubation was continued for 3-4 days and the monolayers were stained with 0.1% crystal violet in 10% formalin neutral buffer solution. Plaques were counted and infectivity titers were expressed as plaque forming unit (pfu)/mL.

Growth curve in cell culture

Subconfluent BHK cells were grown in 24-well plates. Cells were inoculated with each virus at a multiplicity of infection (MOI) of 0.01 pfu. Cells were incubated at 37°C in 5% CO_2. The supernatant was harvested at 12, 24, 48 and 72 h post-inoculation and stored in aliquots at -80°C prior to titration.

Animal model

Viruses were inoculated subcutaneously into 5-week-old female C57BL6 mice (Jackson Immuno Research). Morbidity was defined as the appearance of > 10% weight loss. Surviving mice were monitored for 28 days post-infection to obtain survival curves and mortality rates. For the analysis of viral distribution in tissues, serum, brain, and spleen were collected from the mice on days 3, 5, 7, and 9 post-infection. Organs were individually weighed and homogenized, and prepared as 10% suspensions (w/v) in PBS that contained 10% FCS. The suspensions were clarified by centrifugation (4,000 rpm for 5 min, 4°C) and the supernatants were titrated by plaque assay on BHK cells.

Statistical analysis

RNA copies and viral titers were log transformed and *P*-values were calculated using unpaired Student's *t*-tests.

Results

The Sofjin strain has undergone many passages, resulting in a variety of variants in the viral stock. The direct sequence and cloning of the viral genome revealed four nucleotide substitutions that resulted in amino acid differences in the parental Sofjin strain (Table 1, Fig. 2). To analyze the effect of these amino acid differences on the characteristics of the parental virus, replicons and infectious cDNA clones of the Sofjin strain were constructed as depicted in Figure 2.

Construction of the subgenomic replicon of the Sofjin-HO

The overall strategy to construct the replicon of the TBEV Sofjin strain is outlined in Figure 1A. RNA transcription of replicon RNA was driven by an SP6 promoter, and contained an in-frame deletion in the coding sequence for structural proteins. For correct membrane integration of the non-structural proteins of Sofjin-HO, the coding region for C protein was fused to a C-terminal

fragment of E, which functioned as an internal signal sequence for the subsequent NS1 protein. To analyze the effects of the amino acid differences (Ala or Asp at position 496 in E protein, Met or Thr at position 58 in NS4A protein) derived from the cell culture adaptations, four replicons were constructed (Fig. 2).

To examine the ability of these constructs to replicate and translate viral proteins in cells, BHK-21 cells were transfected with *in vitro*-synthesized RNA, and the expression of virus proteins was analyzed by immunofluorescence at 1 or 4 days post-transfection (Fig. 3). TBEV antigens were detected in the cells transfected with the Sofjin-REP E496-A/4A58-M or Oshima-REP RNA, and the number of TBEV antigen-positive cells increased from 1 to 4 days post-transfection. No or few TBEV antigens were detected in the cells transfected with the other Sofjin-replicon RNAs. At 1 day post-transfection, a smaller number of antigen-positive cells was observed in Oshima-REP than Sofjin-REP E496-A/4A58-M, but there were no differences at 4 days post-transfection.

Intracellular replicon RNAs were determined by real-time PCR at 4 days post-transfection. As shown in Figure 4, more than 10-fold more RNA was detected in cells transfected with Sofjin-REP E496-A/4A58-M and Oshima-REP compared with cells transfected with the Sofjin-REP E496-A/4A58-T, Sofjin-REP E496-D/4A58-M, and Sofjin-REP E496-D/4A58-T. The nucleotide sequences of each replicon RNAs were determined, and no reversions or compensating mutations were observed. These results indicate that the difference in the E position 496 (A/D) and NS4A position 58 (M/T) affected the genome replication.

Construction of the full-length infectious clone of the Sofjin-HO

The full-length infectious cDNA clone, Sofjin-IC, was created by insertion of the coding regions for C, prM and E (nt 240–2291) into Sofjin-REP E496-A/4A58-M (Fig. 1B). In the inserted region of the parental virus genome, there were two amino acid differences derived from cell culture adaptations at prM position 121 (L/F) and E position 122 (E/G/A). To analyze the effects of these amino acid differences, infectious cDNA clones were constructed. Because the Glu-to-Gly or Ala

substitution was previously shown to increase the net positive charge of the E protein by the loss of an acidic residue Glu [38], 4 combinations as depicted in Figure 2 were selected for the generation of recombinant viruses. Recombinant viruses were obtained from the cells transfected with the mRNA of the Sofjin-IC plasmids.

Growth curves of the recombinant viruses were generated to determine how the mutations in Sofjin-IC affected viral production (Fig. 5). No significant difference was observed between the growth curves of the parental Sofjin-HO and each of the Sofjin-IC viruses, indicating that the amino acid differences at prM position 121 and E position 122 did not affect virus growth in BHK cells. Oshima-IC replicated more slowly than Sofjin-HO and Sofjin-IC viruses, and the titer increased at 48 h post-infection to 1/10 of the Sofjin-HO and Sofjin-IC viruses ($p < 0.05$).

Pathogenicity of the recombinant viruses in mouse model

The pathogenicity of the recombinant viruses was examined in a mouse model. Five adult C57BL/6 mice were infected subcutaneously with 10^6 or 10^3 pfu. of each virus and survival was recorded for 28 days (Fig. 6, Table 2). The mice inoculated with each Sofjin-IC virus showed signs of illness, such as reduced body weight, and hindlimb paralysis, as observed in the mice inoculated with the parental Sofjin-HO virus.

With the 10^6 pfu inoculation, Sofjin-IC prM121-F/E122-E and prM121-L/E122-E had similar virulence to the parental Sofjin-HO (100% mortality and mean survival time 8.2-9.4 days). The Sofjin-IC prM121-F/E122-A and prM121-L/E122-G viruses killed three and all five mice, respectively. The day of onset and death was delayed compared with the parental Sofjin-HO and the recombinant virus with glutamic acid at position 122 in the E protein. With the 10^3 pfu inoculation, Sofjin-IC prM121-F/E122-E and prM121-L/E122-E showed similar high virulence to the parental Sofjin-HO, but times of onset and death were slightly shorter than those of Sofjin-HO. The Sofjin-IC prM121-F/E122-A and prM121-L/E122-G viruses killed all and four mice, respectively, and the day of onset and death was delayed as observed in the 10^6 pfu inoculation. In the 10^3 and 10^6 pfu

inoculations, the day of disease onset in the mice inoculated with Oshima-IC was similar to that with Sofjin-HO. However, the survival time was delayed and the mortality was lower than for Sofjin-HO. As previously reported in encephalitis flavivirus [11, 22], dose independent mortality was observed between the 10^3 and 10^6 pfu inoculations of each viruses. However, shorter time of onset and death was observed in most of the mice inoculated with 10^3 pfu of the viruses than in that with 10^6 pfu. These results indicate that the amino acid difference at position 122 in the E protein was important for the virulence of the Sofjin strain.

To examine the correlation between disease development and viral replication in organs, the viral loads in the blood, spleen, and brain were compared in mice inoculated with the Sofjin-IC prM121-L/E122-G, Sofjin-IC prM121-L/E122-E, Sofjin-HO and Oshima-IC viruses (Fig. 7). Transient viremia was observed in the mice infected with each virus, and it almost disappeared by 7 days post-infection. Slight increases in viral replication were observed in the spleen after the viremia (from 5 days post-infection).

After inoculation with Sofjin-IC prM121-L/E122-E, virus was detected in the brain from 5 days post-infection, and the titers reached 7.9×10^7 pfu/mL at 7 days post-infection, whereas virus was not detected in the brain of mice inoculated with Sofjin-IC prM121-L/E122-G by 9 days post-infection. In the Sofjin-HO inoculated mice, virus was first detected at 9 days post-infection, and the titer was 1.2×10^5 pfu/mL. Similar results were obtained from the inoculation of the Sofjin-IC prM121-F/E122-A and prM121-F/E122-E (Supplementary Fig. 1). As observed in the mice inoculated with prM121-L/E122-G, no or only low level virus was detected in the blood, spleen and brain of the mice inoculated with Sofjin-IC prM121-F/E122-A at 7 days post-infection. Similar high level of virus (8.2×10^7 pfu/mL) to Sofjin-IC prM121-L/E122-E was observed in the brain on the mice inoculated with prM121-F/E122-E. In the brain of mice inoculated with Oshima-IC, the virus was first detected at 5 days post-infection, and peaked at 7 days post-infection (2.2×10^4 pfu/mL). However, the replication of the Oshima-IC in brain was lower than that of the Sofjin-IC prM121-L/E122-E ($p < 0.05$ at 5 and 7 days post-infection), indicating that the rapid increase in viral

load in the brain was involved in the different virulence between Sofjin-IC prM121-L/E122-E and Oshima-IC.

These data suggest that viral replication in the brain contributed significantly to the pathogenicity of the Sofjin strain. They also indicated that the amino acid difference at position 122 in the E protein of the Sofjin strain may be important for neuroinvasiveness and the multiplication in the brain.

Discussion

We constructed and characterized replicons and infectious cDNA clones of the Sofjin strain of TBEV. Production of viral proteins and replication of replicon RNA were observed in the replicon-transfected cells (Fig. 3, 4). Infectious viruses were recovered from the infectious cDNA clone (Fig. 5), and mice inoculated with the recombinant viruses showed similar signs of disease, including neurological symptoms, to those inoculated with the parental Sofjin-HO virus (Fig. 6, 7). The amino acid differences derived from the cell culture adaptations of the parental Sofjin-HO were analyzed by using the replicons and infectious cDNA, and it turned out that some of them affected viral characteristics.

The replicons demonstrated two amino acid codon substitutions that were associated with attenuation of viral replication (Ala496 to Asp in the E protein, and Met58 to Thr in the NS4A protein). Residue 496 in the E protein is located just before the recognition site of host signal peptides for NS1 protein in the second transmembrane region of E [9, 10, 12]. It has been reported that the specificity of the signal sequence is important for the correct cleavage, which leads to proper maturation of NS1 protein. The alanine residue at 496 in E protein is highly conserved among most tick-borne flaviviruses. Thus, it is possible that the Ala-to-Asp substitution at residue 496 in the E protein disturbs the correct cleavage. This may cause a functional deficiency in NS1 protein, such as the interaction with other viral components [34] and the formation of the replication complex [33, 40, 41, 50].

NS4A protein is a small, hydrophobic, membrane-associated protein involved in RNA replication. It has been reported that NS4A protein functions as an endoplasmic reticulum (ER)-membrane-associated protein at the assembly of the replication complex in flaviviruses [34, 36, 50], although the exact membrane topology of NS4A protein has yet to be determined. Residue 58 in NS4A protein is located in the conserved hydrophobic residues of the predicted first transmembrane region. It is possible that the Met58 to polar Thr substitution affects the membrane-spanning domain and interaction with other transmembrane domains of NS4A protein or other membrane-associated

viral proteins. A change in membrane-associated-protein topology could lead to a defect in the replication properties of NS4A protein such as the formation of the replication complex by binding with ER membranes.

By analysis of infectious cDNA clones, it was shown that the amino acid differences at position 121 in prM protein did not influence virus growth or virulence in mice. However the amino acid differences at position 122 in the E protein greatly affected virulence in mice. The recombinant virus with Glu at residue 122 in the E protein showed greater neuroinvasiveness than the viruses with Gly or Ala.

The E protein is the major virion surface protein and mediates binding and membrane fusion [35]. E protein consists of head-to-tail homodimers that lie parallel with the virus envelope. Each E subunit is composed of three domains. Residue 122 in E protein is located in the surface of domain II, and the Glu-to-Gly or Ala substitution increases the positive surface charge of the E protein [29, 38]. In several flavivirus studies, it has been reported that multiple passage in cells induce selection of virus variants with an increased positive charge on envelope proteins [3, 7, 14, 28, 29, 38, 43], as observed in the present study. This results in high affinity of the virus for negatively charged substances, such as glycosaminoglycans (GAGs). GAGs are highly sulfated polysaccharides that are present almost ubiquitously on cell surfaces [1, 4, 26, 49]. Previous studies have shown that GAG-adapted viruses have reduced virulence in animals, and our previous results have demonstrated that TBEV with increased affinity for GAGs is cleared more rapidly from the blood and organs [14]. The results of the present study are consistent with the previous studies and indicated that increased positive charge, induced by amino acid substitution, reduced neuroinvasiveness, leading to the lower virulence.

With a the low dose (1,000 pfu) of parental Sofjin-HO, the onset of disease and death was delayed compared with that with the recombinant virus with Glu at position 122 in the E protein, although both viruses showed 100% mortality. With Sofjin-HO inoculation, the virus was first detected at 9 days post-infection, whereas Sofjin-IC prM121-L/E122-E entered the brain earlier (5

days post-infection). This could have been due to the low proportion of the viruses with Glu at position 122 in E protein in the parental Sofjin-HO. Sequence analysis revealed that only the virus with Glu at the residue 122 in the E protein was recovered from the brain of the mice infected subcutaneously with Sofjin-HO. These data indicate the importance of residue 122 of the E protein in the virulence of Sofjin-HO in a mouse model.

In the comparison between the Sofjin and Oshima strains, lower replication efficacy of Oshima than Sofjin was observed in the replicons and infectious cDNA. This was consistent with our previous data using the parental viruses [13]. Furthermore, it was shown that the virulence of Oshima-IC, which has Glu at residue 122 in the E protein, in mice was lower than that of Sofjin-IC, also with Glu at position 122 in E the protein. No difference was observed between peripheral multiplication and the neuroinvasiveness of the two viruses, but growth in the brain of the Sofjin-IC virus was greater than that of the Oshima-IC virus. These results indicate that the amino acid differences between the two viruses contributed to the different characteristics of the Far-Eastern subtype of TBEV.

In summary, we constructed replicons and infectious cDNA clones of the TBEV Sofjin strain and demonstrated their utility in research of TBEV replication and pathogenesis. We have also identified the amino acid differences in the E and NS4A proteins that were important for RNA synthesis and virulence of the parental Sofjin-HO strain. The replicons and infectious cDNA clones constructed in this study could be useful in future studies to reveal the viral molecular determinants that are involved in the replication and pathogenicity of TBEV.

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Figure legends

Fig. 1. Schematic representation of TBEV genome, replicon, and infectious cDNA constructs. **(a)** Three fragments were cloned into the low-copy plasmid pGGV$_{S209}$. Sofjin replicon regions were inserted under the control of the SP6 promoter. A *Spe*I restriction endonuclease site was fused to the 3'-end of the viral genome. **(b)** To construct infectious cDNA, the coding regions for C, prM, and E were inserted into Sofjin-REP plasmid.

Fig. 2. Nucleotides and amino acids difference between Sofjin-HO, Sofjin-IC, and Sofjin REP. Bold type indicate the sequence registered in GeneBank (Accession No. 062064)

Fig. 3. Detection of TBEV antigen in BHK cells transfected with the *in vitro* transcribed replicon RNA. BHK cells were electroporated with mRNA of Sofjin-REP E496-A/4A58-M (i and viii), E496-A/4A58-T (ii and viii), E496-D/4A58-M (iii and ix), E496-D/4A58-T (iv and x), Oshima-REP (v and xi), amd mock (vi). At 1 day **(a)** or 4 days **(b)** post-transfection, viral proteins were visualized by immunofluorescence with anti-TBEV antibodies.

Fig. 4. Real-time PCR for quantification of replication of replicon RNA. Total RNA was extracted from the cells electroporated with each replicon RNA at 4 days post-electroporation. Replicon RNA was quantified using the TaqMan real-time PCR assay. Error bars represent the SD (n=3). The data were subjected to a *Student's t-test* statistical analysis. † and ‡ denote $p < 0.05$ and $p < 0.01$ respectively.

Fig. 5. Comparison of growth curves of parental strain Sofjin-HO (closed triangles), Sofjin-IC prM121-F/E122-A (closed diamonds), Sofjin-IC prM121-F/E122-E (open triangles), Sofjin-IC prM121-L/E122-G (open diamonds), Sofjin-IC prM121-L/E122-E (open circles) and Oshima-IC (closed squares) viruses. BHK cells were infected with the individual virus at MOI of 0.01 and

supernatant was harvested at 12, 24, 48, and 72 h post-infection. Virus titer was determined by plaque assay in BHK cells. Error bars represent the SD (n=3). * denote the significant difference between Oshima-IC and the other viruses ($p < 0.05$).

Fig. 6. Survival of mice inoculated with Sofjin-IC and Oshima-IC virus. Mice were inoculated subcutaneously with 10^6 **(a)** or 10^3 **(b)** pfu of parental Sofjin-HO (closed triangles), Sofjin-IC prM121-F/E122-A (closed diamonds), Sofjin-IC prM121-F/E122-E (open triangles), Sofjin-IC prM121-L/E122-G (open diamonds), Sofjin-IC prM121-L/E122-E (open circles) and Oshima-IC (closed diamonds) viruses.

Fig. 7. Virus replication in organs. Mice were infected with 1,000 pfu of each virus (parental Sofjin-HO, Sofjin-IC prM121-L/E122-G, Sofjin-IC prM121-L/E122-E, and Oshima-IC). Virus titers in blood **(a)**, spleen **(b)**, and brain **(c)** at the indicated days after infection were determined by plaque assays. The horizontal dashed lines indicate limits of detection for the assay (250 pfu/mL in blood, and 1,000 pfu/mL in spleen and brain). Error bars represent the SD (n=3) *By 9 days post-infection, all mice inoculated with Sofjin-IC prM121-L/E122-E and one mouse inoculated with Sofjin-IC prM121-L/E122-G died.

Fig.1

TBEV genome

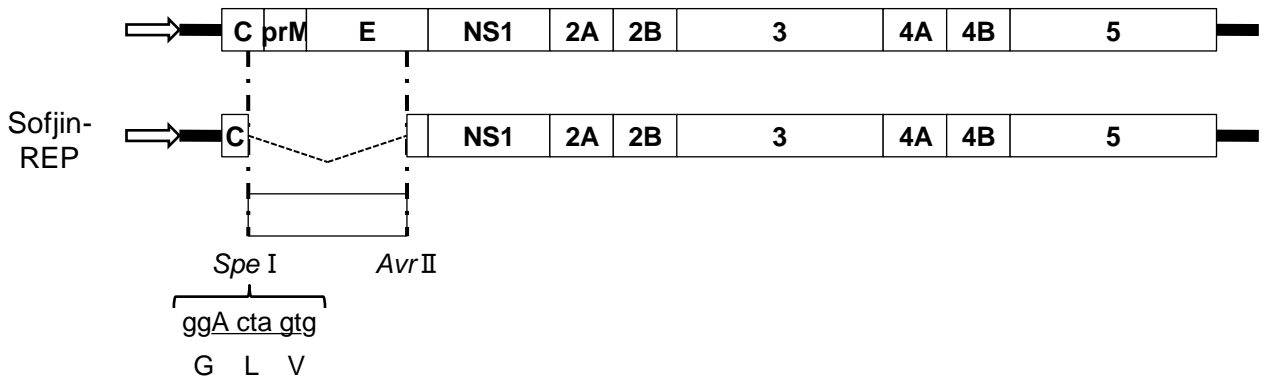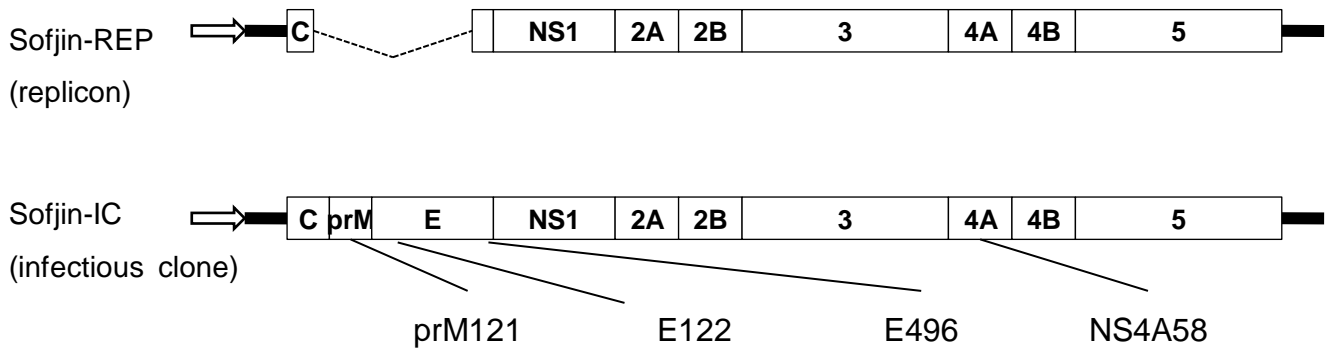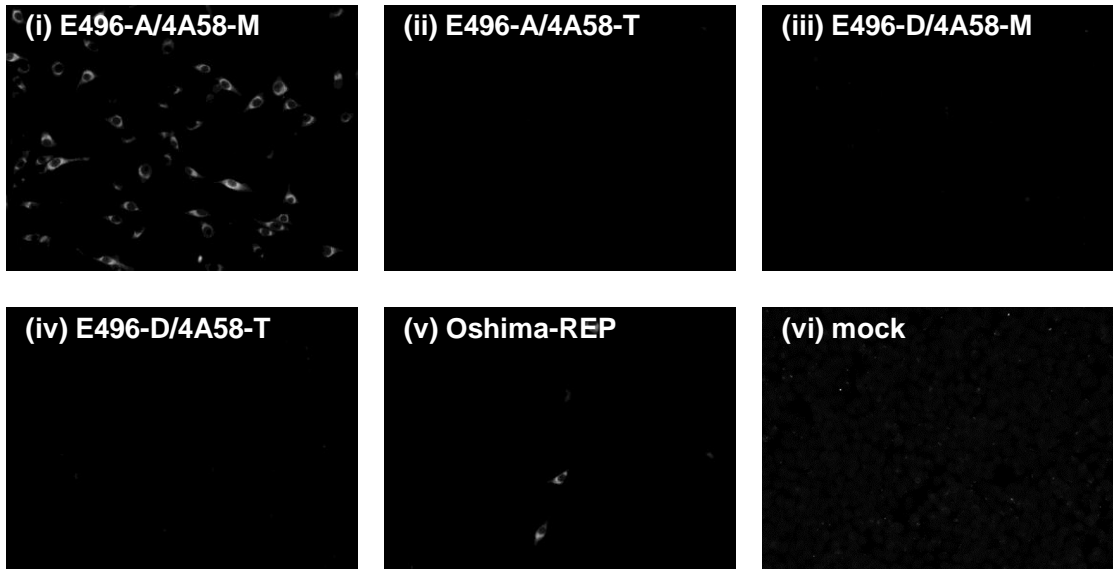(a) Sofjin-REP (replicon)
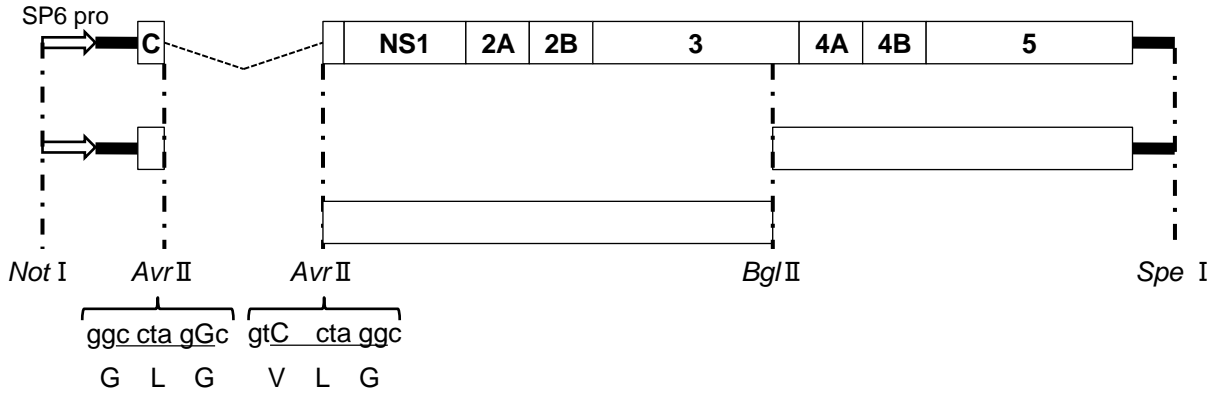
(b) Sofjin-IC (infectious clone)

Fig.2

Sofjin-REP (replicon)

Sofjin-IC (infectious clone)

prM121　E122　E496　NS4A58

	prM121	E122	E496	NS4A58
Sofjin-HO (nucleotide)	Leu/Phe (**c**tc/ttc)	Glu/Ala/Gly (ga**g**/gcg/ggg)	Ala/Asp (g**c**t/gat)	Met/Thr (a**t**g/acg)
Sofjin-REP E496-A/4A58-M	-	-	Ala	Met
Sofjin-REP E496-A/4A58-T	-	-	Ala	Thr
Sofjin-REP E496-D/4A58-M	-	-	Asp	Met
Sofjin-REP E496-D/4A58-T	-	-	Asp	Thr
Sofjin-IC prM121-F/E122-A	Phe	Ala	Ala	Met
Sofjin-IC prM121-F/E122-E	Phe	Glu	Ala	Met
Sofjin-IC prM121-L/E122-G	Leu	Gly	Ala	Met
Sofjin-IC prM121-L/E122-E	Leu	Glu	Ala	Met

Fig.3

(a) 1 day post-transfection

(i) E496-A/4A58-M

(ii) E496-A/4A58-T

(iii) E496-D/4A58-M

(iv) E496-D/4A58-T

(v) Oshima-REP

(vi) mock

(b) 4 day post-transfection

(vii) E496-A/4A58-M

(viii) E496-A/4A58-T

(ix) E496-D/4A58-M

(x) E496-D/4A58-T

(xi) Oshima-REP

Fig.4

Fig.5

Virus titer (log10 pfu/ml) — vertical axis, 3 to 10

Time post-infection (hours) — horizontal axis, 0 to 80

*

Legend:
- —▲— Sofjin-HO
- —◆— Sofjin-IC prM121-F/E122-A
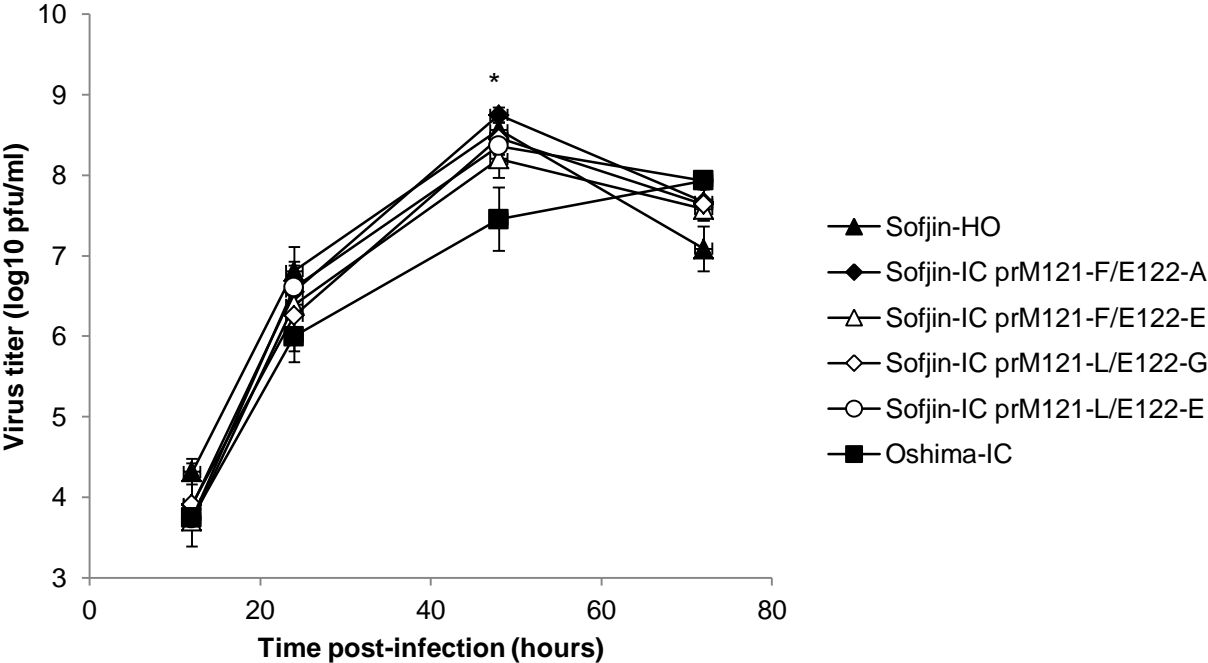- —△— Sofjin-IC prM121-F/E122-E
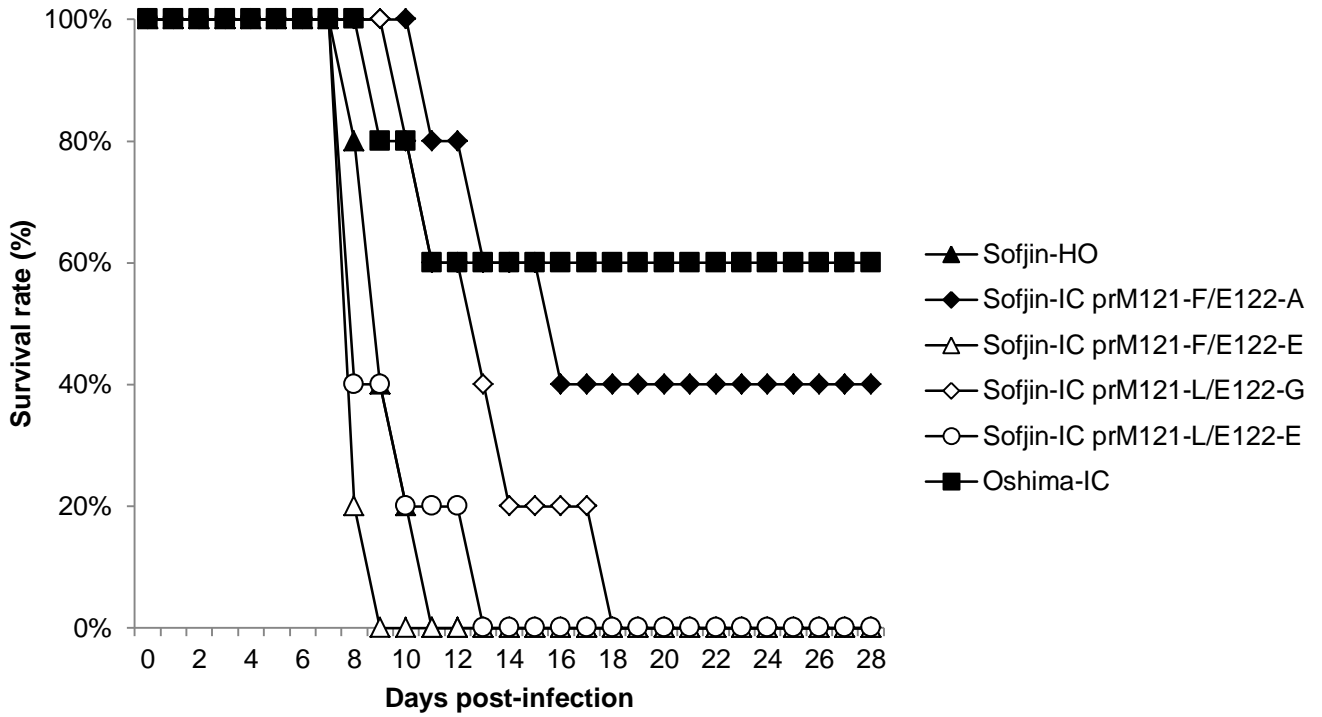- —◇— Sofjin-IC prM121-L/E122-G
- —○— Sofjin-IC prM121-L/E122-E
- —■— Oshima-IC
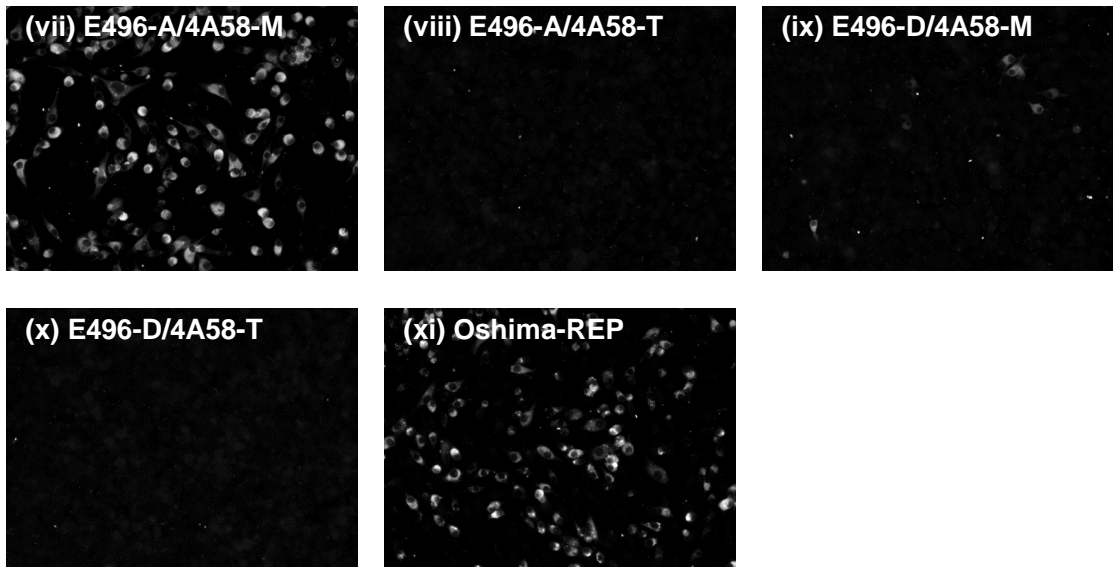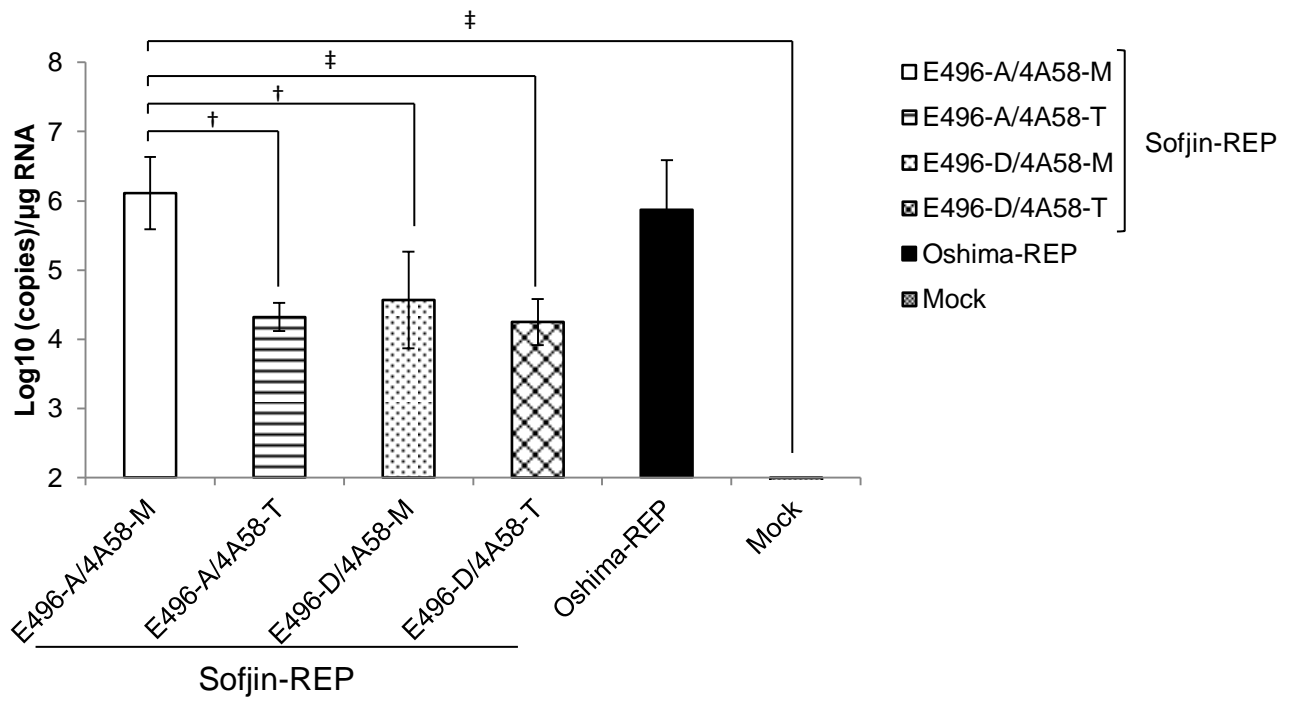
Fig.6

(a) 10^6 pfu

(b) 10^3 pfu

Fig.7

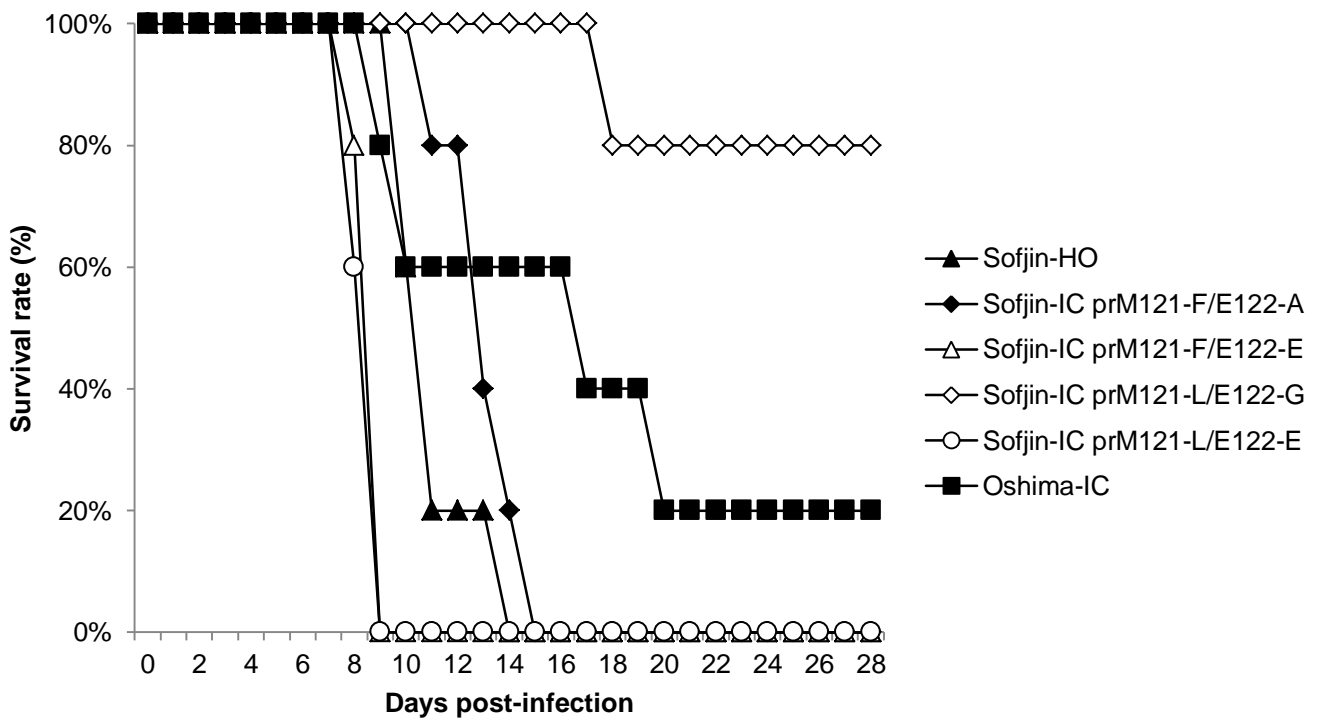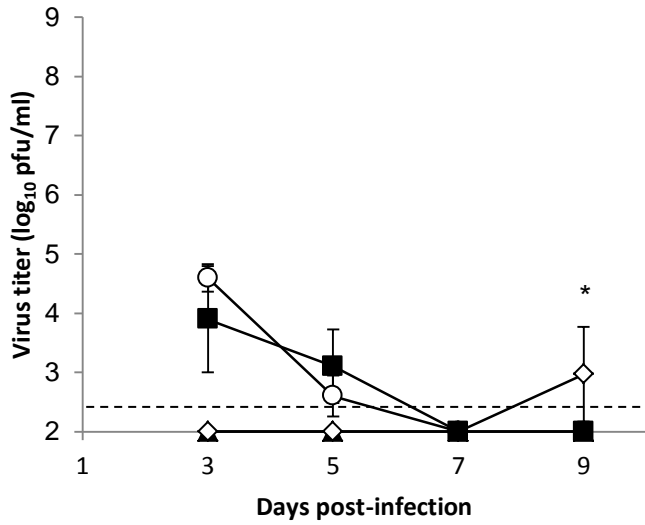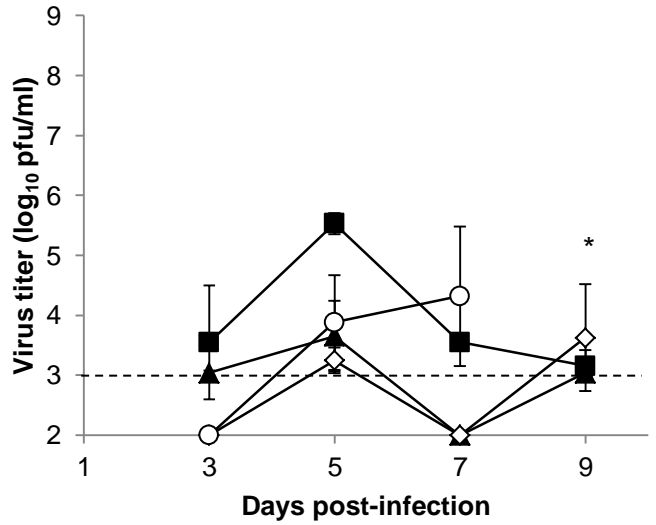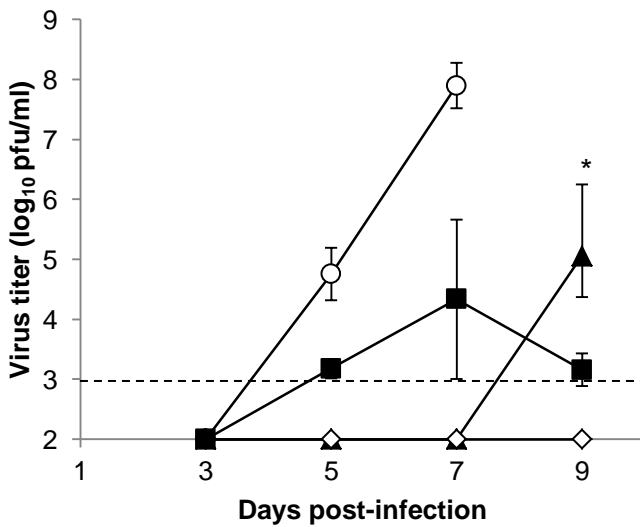## (a) blood

(b) spleen

(c) brain

- Sofjin-HO
- Sofjin-IC prM121-L/E122-G
- Sofjin-IC prM121-L/E122-E
- Oshima-IC

Table. 1 Sequence differences in the parent Sofjin-HO virus

Position	Sofjin-HO		Amino acid
	nt[*1]	aa[*2]	position
780	**C**/T[*3]	L/F	prM121
1336	**A**/C/G	E/A/G	E122
2458	**C**/A	A/D	E496
3528	**C**/T	-(silent)	
4031	**G**/A	-	
4475	**A**/G	-	
4898	**C**/T	-	
5543	**T**/C	-	
6197	**G**/A	-	
6634	**T**/C	M/T	NS4A58
7004	**C**/T	-	
7007	**T**/C	-	
7010	**T**/C	-	
7013	**C**/T	-	
8561	**C**/T	-	
8600	**T**/C	-	
8927	**A**/T	-	
9201	**C**/T	-	

[*1] The nucleotides which showed double or triple waves in the direct sequence of the viral genomic RNA.

[*2] The amino acid differences were confirmed by the cloning of the viral genome into the cloning vectors, in addition to the direct sequence. Each nucleotides were observed in more than 20 % of the cloned plasmids.

[*3] Bold type indicate the sequence registered in GeneBank (Accession No. 062064)

Table. 2 Physical differences between mice infected with Sofjin-IC viruses, parental Sofjin-HO and Oshima-IC virus
Five adult mice C57BL/6J was infected with indicated titer of each viruses subcutaneosly.

	10^6 p.f.u./mouse s.c.				10^3 p.f.u./mouse s.c.			
	onset of disease (day)	survival time (day)	morbidity (%)	mortality (%)	onset of disease (day)	survival time (day)	morbidity (%)	mortality (%)
Sofjin-HO	8.4±1.34	9.4±1.14	100	100	9.6±1.14	11.2±1.64	100	100
Sofjin-IC prM121-F/E122-A	11±1	13.33±2.51	60	60	11.4±1.52	13.2±1.48	100	100
Sofjin-IC prM121-F/E122-E	7	8.2±0.45	100	100	7±0.71	8.8±0.45	100	100
Sofjin-IC prM121-L/E122-G	11.2±2.17	13.2±3.11	100	100	16	18	20	20
Sofjin-IC prM121-L/E122-E	6.6±0.55	9.4±2.19	100	100	7.4±0.55	8.6±0.55	100	100
Oshima-IC	8±0.58	10±1	80	40	9.2±0.73	14±2.68	100	80