

Original article

Pathologic Potential of Variant Clones of the Oshima Strain of Far-Eastern Subtype Tick-Borne Encephalitis Virus

Le Xuan Luat, Mya Myat Ngwe Tun, Corazon C. Buerano, Kotaro Aoki, Kouichi Morita and Daisuke Hayasaka*

Received 28 September, 2013 Accepted 20 October, 2013 Published online 4 February, 2014

Abstract: Tick-borne encephalitis virus (TBEV) is a zoonotic agent that causes acute central nervous system (CNS) disease in humans. We previously suggested that immune response in addition to CNS infection contribute to mouse mortality following TBEV infection. However, we did not examine the influence of virus variants in the previous study. Therefore, in this study, we investigated the biological and pathologic potentials of the variant clones in the TBEV Oshima strain. We isolated eight variant clones from the stock virus of the Oshima 5-10. These variants exhibited different plaque morphologies in BHK cells and pathogenic potentials in mice. Full sequences of viral genomes revealed that each of the variant clones except one had specific combinations of nucleotide and amino acid changes at certain positions different from the parent strain. We also showed that an amino acid substitution of Glu₁₂₂→Gly in the E protein could have affected virus infection and replication *in vivo*, as well as the attenuated pathogenicity in mice. These data confirm the presence of virus variants or quasispecies from the parent strain. Further elucidation of the effect of each variant clone on immune responses such as the T-cell response is an important priority in the development of an effective vaccine and treatment strategies for tick-borne encephalitis.

Key words: Far-eastern subtype TBEV, variant clones, E protein

INTRODUCTION

Tick-borne encephalitis virus (TBEV) is a causative agent of acute central nervous system (CNS) disease in humans [1, 2]. TBEV is a member of the family *Flaviviridae*, genus *Flavivirus*, whose genome encodes three structural proteins (C, prM and E) and five non-structural (NS) proteins (NS1, NS2, NS3, NS4 and NS5) [2–4].

TBEV is prevalent over a wide area of Europe and Asia, and is divided into three subtypes comprising the European (Eu-), Siberian and Far Eastern (FE-) subtypes [5, 6]. It has been suggested that the FE-TBEV is associated with a disease more severe than the one caused by the other subtypes [2]. TBEV can cause tick-borne encephalitis (TBE) which is a potentially fatal neurological infection affecting humans [4]. The virus can infect humans through the bite of an infected tick. The TBEV endemic areas of Europe and Asia correspond to the geographical distribu-

tion of *Ixodes* tick species [1, 7–11].

In human cases, the neurological symptoms include fever, headache, meningitis, meningoencephalitis and meningoencephalomyelitis [1]. However, the clinical features are not unique to TBE, and laboratory diagnosis is required to distinguish it from other neurological disorders [3, 12, 13]. Although clinical symptoms vary from febrile illness to meningitis and encephalitis, the mechanism of severe encephalitis has not been fully elucidated.

The laboratory mouse model is commonly employed to elucidate the mechanism of disease development following TBEV infection *in vivo*. [14–17]. Recent works have proposed that CNS pathology following TBEV infection is a consequence of viral infection of the corresponding cells and the resulting inflammatory response [18].

Using a mouse model of infection with the Oshima strain of FE-TBEV, we have shown that immune and stress responses in addition to CNS infection contribute to mouse

Department of Virology, Institute of Tropical Medicine, Nagasaki University

*Corresponding author:

Department of Virology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

Tel: +81-95-819-7829

Fax: +81-95-819-7830

E-mail: hayasaka@nagasaki-u.ac.jp

Affiliation: Department of Virology, Institute of Tropical Medicine, GCOE program, Leading Graduate School Program, Nagasaki University, Nagasaki, Japan

mortality [19, 20]. These results indicated that the host immune response is likely to be a determinant of clinical outcome in this model [19–22]. However, we did not examine the influence of virus variants (quasi-species) in the mouse model experiments regarding TBEV Oshima infection.

Variants of TBEV may induce different degrees of pathology. Previous papers have reported that the amino acid mutations in the E protein of this virus exert an effect on its virulence and neuroinvasiveness [23–25]. For example, Mandl et al. reported that the E protein of the Neudoerfl strain of Eu-TBEV had several different mutations such as Glu₁₂₂→Gly, Ser₁₅₈→Arg, Gly₁₅₉→Arg and Glu₂₀₁→Lys after several passages in cultured cells. These mutations affected the binding sites of the protein and resulted in the attenuation of the virus *in vivo* [26]. It was also shown that neurovirulence of the Oshima strain of FE-TBEV was attenuated by the mutation at position 1579 (A→G, Asp₄₈₃→Gly) [23]. Thus, in this study, our purpose was to investigate the pathologic potential of the variants or quasispecies of the Oshima strain of FE-TBEV in a mouse model.

METHODS

Virus and cell

Stock virus of the Oshima 5-10 strain of TBEV was prepared from infected cell culture medium of baby hamster kidney (BHK) [19]. The BHK cells were grown in Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co.) containing 10% fetal calf serum (FCS). All experiments using live TBEV were performed in a biosafety level 3 laboratory at the Institute of Tropical Medicine, Nagasaki University according to the standard Biosafety Level 3 guidelines.

Plaque assay

Virus titers were determined by plaque forming assays in BHK cells [19]. Briefly, monolayers of BHK cells were grown in 24-well plates and infected with serial dilutions of the virus in culture medium. After incubation at 37°C for 90 minutes, culture medium containing 1% methyl cellulose was added to each well. Cells were then incubated for four days, after which, the medium was removed and cells were stained with crystal violet. Viral titers were expressed as pfu/ml of the diluted viruses in culture medium.

Cloning of Oshima 5-10 by limiting dilution

BHK cells were grown in four 96-well plates and then inoculated with Oshima 5-10 at a concentration of 0.1 pfu/well. Cytopathic effects (CPE) were observed in less than 10 wells per plate. The infected culture fluids in these wells were harvested and inoculated in fresh BHK cells, and the

procedure of identifying CPE and inoculation in BHK cells was repeated. Finally, the stock viruses of each clone were prepared from cell culture medium of BHK.

Mice

C57BL/6j (B6) mice were purchased from Japan SLC Corporation. Five-week old female B6 mice were subcutaneously inoculated with 10⁴ PFU of TBEV Oshima 5-10 parent (Oshima-pt) and Oshima clones diluted in 200 µl of EMEM containing 2% FCS. Mock-infected mice were inoculated with the supernatant medium of uninfected BHK cells. The mice were weighed daily and observed for clinical signs. The animal experiments were performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology. The experimental protocols were approved by the Nagasaki University Animal Care and Use Committee (approval number: 091130-2-7/0912080807-7).

Virus titrations

At nine days post-infection (p.i.) with 10⁴ PFU of TBEV Oshima clones, mouse brains and spleens were collected after perfusion with cold PBS. The spleens were immediately immersed in RNAlater (Ambion). The collected tissues were stored at –80°C until they were used.

The brains were homogenized in ten volumes of PBS containing 10% FCS and diluted with EMEM with 2% FCS. Virus titers were determined by plaque-forming assays in BHK cells and were expressed as pfu/g of tissue.

Total RNA was extracted from spleens using RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. The levels of viral RNA were measured by real time-PCR as demonstrated previously [27, 28]. The copy numbers were calculated as a ratio of the copy numbers in 100 ng of RNA.

Genome sequence of the Oshima 5-10 clones

Viral RNA of each Oshima 5-10 clone was extracted from the cell culture fluids of each stock virus using QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol. Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen) and random hexamers. PCR was performed to cover the whole genome sequence using TAKARA Ex Taq DNA polymerase (TAKARA BIO Inc.). The cycle sequencing reaction was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies), and the DNA sequence was determined in the Applied Biosystems 3730 DNA Analyzer (Life Technologies).

Primers were designed based on the genome of Oshima 5-10 strain (GenBank Acc. No. AB062063.2). Sequence was performed in both directions with specific primer corresponding to each fragment. Sequence results were analyzed with MEGA5 [29] and Unipro Ugene [30]. The nucleotides sequences were aligned by ClustalX 2.1 [31] for full genome analyses.

3D structure of E protein of TBEV

The 3D structure of E protein was shown using the PyMOL Molecular Graphics System, Version 1.6 Schrödinger, LLC.

RESULTS

Clones of the Oshima 5-10

We isolated and examined eight clones of Oshima 5-10 virus and designated them as Oshima-A4, A9, A11, B11, C1, E2, E3, and E7. The clones exhibited a different morphology of plaques in BHK cells (Fig. 1). Oshima-pt showed various sizes of plaques (Fig. 1). The plaque size of Oshima-A4, A9 and B11 was comparatively small, while that of C1 was large (Fig. 1). However, Oshima-A11, C1, E2, E3, and E7 virus caused a mixture of large and small plaques (Fig. 1). These results indicate that parent Oshima

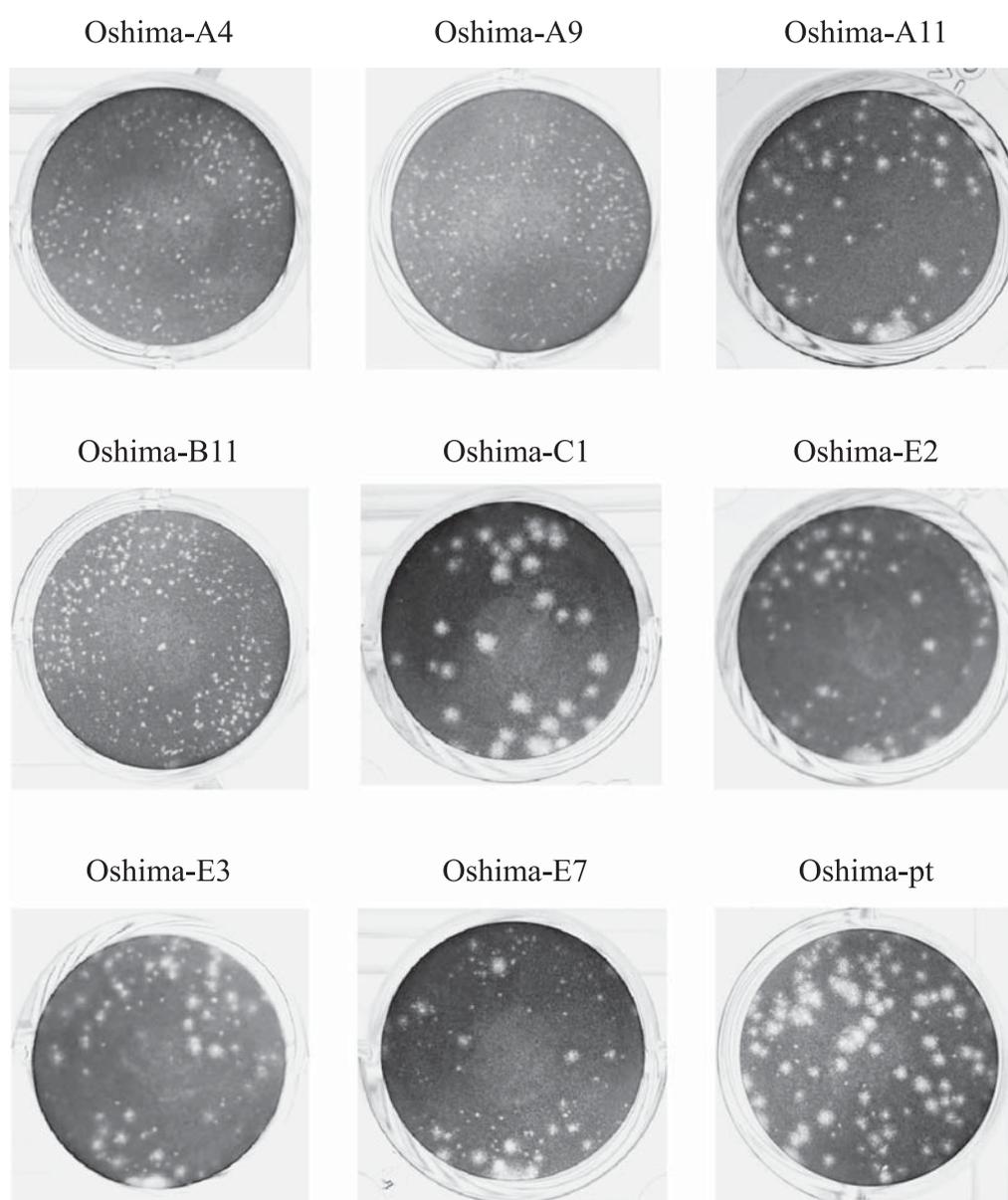


Fig. 1. Plaque morphology of TBEV Oshima clones. Oshima-A4, A9 and B11 exhibited small plaques, while Oshima-C1 showed large plaques. Oshima-A11, E2, E3, E7 and Oshima-pt showed a mixture of small and large plaques.

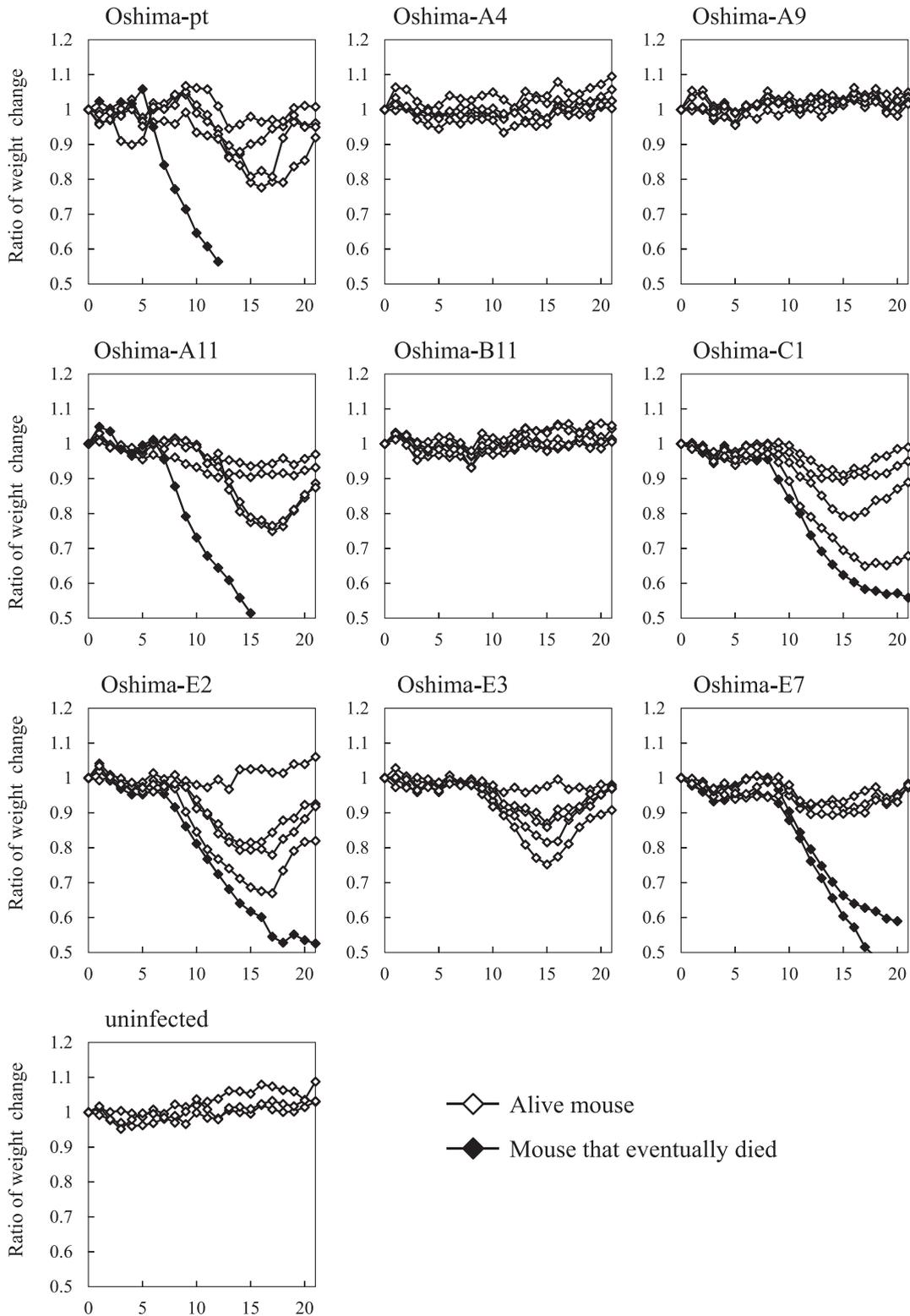


Fig. 2. Weight changes in mice following subcutaneous inoculations with TBEV Oshima clones. Five mice in each group were subcutaneously inoculated with TBEV Oshima clones and observed for 21 days. Mouse weight was observed daily and compared with that at day 0. Each mouse is represented by a line that connects the open or closed diamond-shaped boxes. Each box represents the observed ratio of weight change at observation day. The lines with the open and closed boxes indicate a living mouse and a mouse that eventually died during the observation period, respectively.

5-10 virus consists of quasi-species based on the various sizes of plaques exhibited by the clones.

Pathogenicity of TBEV Oshima clones in mice

To examine the pathogenicity of TBEV Oshima clones in mice, we subcutaneously inoculated each clone in five corresponding B6 mice, which were then observed for morbidity (weight loss) and mortality. All Oshima-pt-infected mice exhibited weight loss and one mouse died at 12 days p.i. (Fig. 2). This observation was reproducible as in our previous study [19]. Oshima-A11, C1, E2 and E7 also caused weight loss in 4-5 mice and the death of 1-2 mice within 21 days p.i. similar to that of the parent virus (Fig. 2). Four Oshima-E3-infected mice exhibited weight loss and all mice survived (Fig. 2).

On the other hand, the small plaque-forming clones such as Oshima-A4, A9 and B11 did not cause any weight loss, clinical signs or death similar to the observations in the uninfected mice (Fig. 2).

These results indicate that only the clones producing small plaques, had attenuated virulence in mice and that the other Oshima clones with plaque-forming characteristics similar to those of the parent strain shared the same pathogenic potential as the parent strain.

Virus infections in brains and spleens of Oshima clones-infected mice

To observe the virus infection in the CNS, we investigated the infectious viral loads in the brains of Oshima clone-infected mice. Following inoculation with Oshima-pt, all mice exhibited more than 10^4 pfu/g of tissue in the brain (Fig. 3A). Oshima-A11-, C1- and all E7-infected mice also showed more than 10^4 pfu/g of tissue in the brain (Fig. 3A). Following Oshima-E3 infection, one mouse showed a viral load in the brain, but the other two mice did not (Fig. 3A). The viral load in the brain of all Oshima-A4-, A9- and B11-infected mice was under the detection limit (Fig. 3A).

Next, we confirmed peripheral infections in Oshima clone-infected mice. We employed quantitative RT-PCR to detect viral RNA in the spleen instead of using plaque assay, because no infectious virus could be detected by this technique at nine days p.i. In all Oshima-pt-, A11-, C1-, E2- and E7-infected mice, viral RNA was detected, indicating that peripheral infection and replication occurred in these mice (Fig. 3B). In Oshima-E3-infected mice, one mouse showed peripheral infection, but no viral RNA was detected in the spleen of the other two mice (Fig. 3B). The viral RNA level was under the detection limit in two of three Oshima-A4- and B11- and in all of the A9-infected mice (Fig. 3B).

It is noteworthy that the Oshima-A4-, A9- and B11-

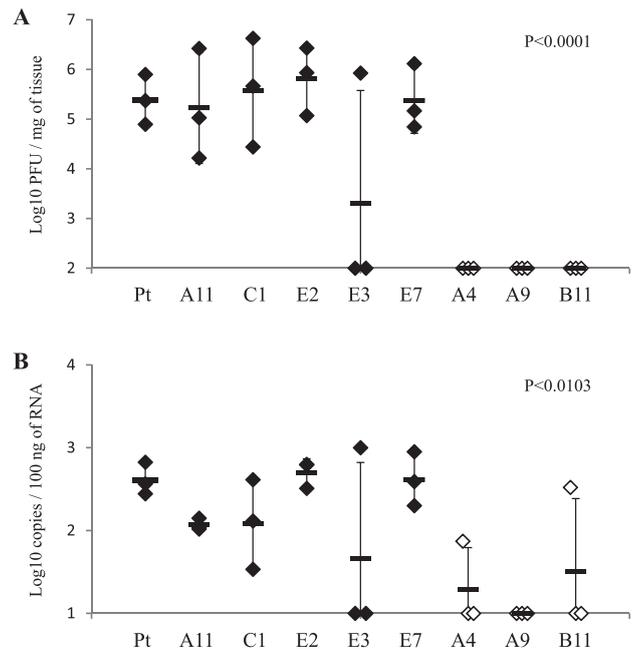


Fig. 3. Viral load in brains and spleens. Three mice per group were subcutaneously infected with 10^4 PFU of TBEV Oshima clones, and their brains and spleens were collected at day 9 p.i.. (A) Infectious viral load in the brain. (B) Copy numbers of TBEV genome in the spleen. Oshima-pt, A11, C1, E2, E3 and E7, which showed a weight reduction in the infected mice, are indicated by closed diamond boxes. Oshima-A4, A9 and B11, which showed no weight reduction in the infected mice, are indicated by open diamond boxes. Error bar: SD, P: ANOVA.

infected mice with levels of viral RNA under the detection limit in the spleen did not exhibit viral infections in the brain either, indicating that viral infections did not occur or were very limited. These results suggest that Oshima-A4, A9 and B11 viruses exhibited very limited peripheral and CNS infection, and had an attenuated virulence in mice.

Genome sequence of Oshima 5-10 clones

We determined the full genome sequence of Oshima 5-10 clones by the direct sequence method. Each clone showed a few differences in nucleotide and amino acid at certain positions compared with Oshima-pt (Table 1). Interestingly, the Oshima-A4, A9 and B11 that showed small plaques and attenuated virulence had a common nucleotide substitution of A to G at position 1342 at the E-coding region, and it resulted in the substitution of amino acid from glutamic acid to glycine at position 122 of the E protein (Table 1). This substitution was not detected in Oshima-A11, C1, E2, E3 or E7 (Fig. 2). These results suggest that an amino acid change of glutamic acid to glycine in E protein

Table 1. Nucleotide and amino acid changes in TBEV Oshima 5-10 clones with respect to the parent virus.

Gene	prM	E	E	E	E	E	E	NS1	NS1	NS3	NS3
Nucleotide (amino acid) position	916 (149)	1277 (101)	1342 (122)	1415 (148)	1544 (191)	1644 (224)	2246 (425)	2569 (36)	2894 (130)	5614 (338)	5652 (344)
Oshima-pt	T (V)	T (G)	A (E)	G (G)	T (G)	C (L)	T (S)	A (E)	A (P)	C (S)	G (D)
Oshima-A4	—	—	G (G)	—	—	—	C (S)	—	—	—	—
Oshima-A9	—	—	G (G)	T (G)	C (G)	—	—	—	—	—	—
Oshima-A11	C (A)	—	—	—	—	—	—	T(V)/A(E)	—	—	—
Oshima-B11	—	—	G (G)	—	—	—	—	—	—	T (F)	—
Oshima-C1	—	G (G)	—	—	—	—	—	—	G (P)	—	T (Y)
Oshima-E2	—	—	—	—	—	—	—	—	—	—	—
Oshima-E3	—	—	—	—	—	T(L)/C(L)	—	—	—	—	—
Oshima-E7	—	—	—	—	—	—	—	—	—	—	T (Y)

*capital letter in parentheses refers to amino acid

affects the plaque morphology and may influence the biological activity of the Oshima strain of FE-TBEV.

DISCUSSION

In this study, we examined eight variant clones from the stock virus of Oshima 5-10, a strain of FE-TBEV. These variants exhibited different pathogenic potentials in mice. Full sequences of viral genomes revealed that each of the variant clones except one had a specific combination of nucleotide and amino acid changes at certain positions different from the parent strain. These data confirm the presence of virus variants or quasispecies from the parent strain.

Oshima-A4, A9 and B11 exhibited attenuated virulence, whereas the others showed pathogenicity similar to that of the parent virus. None of the cloned variants in this study showed a significantly (log rank test) higher virulence than the parent virus, but in another study [14] Sofjin strain surpassed the parent strain in virulence. We also found that attenuated variants had a common amino acid substitution in E protein (Glu₁₂₂→Gly). Mice inoculated with these variants exhibited no CNS viral entry and had very limited peripheral infections. Using the Neudoerfl strain of Eu-TBEV, Mandl et al. reported that the same substitution of Glu₁₂₂→Gly in E protein appeared during adaptation in BHK-21 cells and that the amino acid substitution caused a reduction of virus virulence in mice. Thus, the Glu₁₂₂→Gly amino acid substitution could have affected virus infection and replication *in vivo* and the pathogenicity of both Eu-TBEV and FE-TBEV in mice.

The E protein of flaviviruses is classified as a class II virus membrane fusion protein and plays an important role in receptor binding and fusion of the virus membrane with the host cell membrane [32–34]. At neutral pH, E protein of

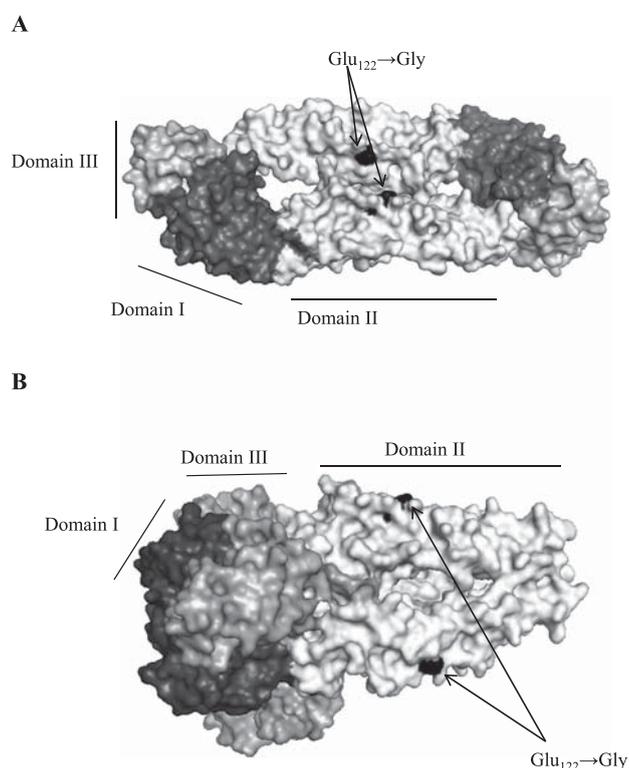


Fig. 4. Position of the amino acid substitution Glu₁₂₂→Gly in E protein. The amino acid at position 122 of E protein is glutamic acid (E), and it is located at domain II of E protein. (A). Dimer structure of E protein of TBEV Oshima 5-10 strain. (B). Trimer structure of E protein of TBEV Oshima 5-10 strain. Arrows point to the area of amino acid substitution from Glu₁₂₂→Gly.

mature flavivirus virion is a homodimer (Fig. 4A) and is located on the virus surface. Each monomer contains three different β -barrel domains, namely domain I (DI), domain

II (DII), and domain III (DIII) (Fig. 4A). DI is a structural domain, contains N-terminal and elongates with DII by a fusion loop (fp) at the tip of DII. This fp is buried through the interaction with a hydrophobic pocket between DI and DIII. DI connects to DIII by a single polypeptide linker region. DIII is an immunoglobulin-like domain and connects to the C-terminal stem region and transmembrane (TM) domain [35, 36]. At a low pH condition, the homodimer of E protein dissociates, and the fp is exposed and interacts with cell membrane [37]. At the next step, the monomer rearranges and associates into homotrimer (Fig. 4B), which then initiates hairpin formation. DIII and the stem region fold back towards the fusion loop as hemifusion [33]. Finally, the viral membrane completely fuses with the cell membrane through mixing of the outer membrane leaflet (viral membrane leaflet) and the inner membrane leaflet (cell membrane leaflet) and the opening of a fusion pore [38].

The substitution in E protein (Glu₁₂₂→Gly) is located in DII of E protein adjacent to the hinge region (Fig. 4A and 4B). During the membrane fusion process, the dissociation of homodimer and the consequent formation of homotrimer locates Glu₁₂₂→Gly on the surface of the homotrimer structure (Fig. 4B). DII of E protein contains fusion peptide that functions as an inert anchor or active center in the process of fusion between the viral envelope and cell membrane [39]. Mandl *et al.* showed that this substitution acquired potential HS-binding in this position and increased TBEV infectivity in BHK cells [26, 40]. They also showed that this substitution caused attenuation of virulence in mice, although the precise mechanism of the attenuation was not fully elucidated. In this study, we showed that Oshima-A4, A9 and B11 viruses exhibited small plaque size and attenuated virulence in mice. Attenuated virus replications of Oshima-A4, A9 and B11 might also be affected by HS binding [26].

In this study, twelve nucleotide substitutions including the nucleotide that resulted in Glu₁₂₂→Gly were detected. It is noteworthy that Oshima-E3 exhibited attenuated virulence and one nucleotide change in E protein but that the change did not result in amino acid change. However, at this stage of our knowledge, it is difficult to explain how these substitutions affect viral biology and virulence. Information on these substitutions has not been reported previously. Thus, investigations regarding the role of these nucleotide substitutions may provide important information in understanding the biology and pathogenesis of TBEV.

The laboratory mouse model has been commonly employed to study the pathology of encephalitic flaviviruses. Using the mouse model, we previously showed that the Oshima strain of FE-TBEV caused variation of fatal outcomes and we suggested that the immunopathogenic pro-

cess contributes to severe disease and mortality [19]. Furthermore, we showed that specific T-cell receptor (TCR) repertoires were present in dying mice during TBEV infection [41]. These data raise the possibility that there may be a variety of specific T-cell clones affecting either protective or pathogenetic functions in dying and recovering mice, indicating that the differentiation of specific T-cell clones is one of the key factors of the disease. However, the mechanism by which the differentiated T-cell clones appeared and developed remained unclear. Therefore, the variant amino acid of the variant clones found in this study might exert an effect on the various T-cell epitopes and on the differentiated T-cell clones in individual mice. Thus, the data from the present study could provide an important clue for understanding the variations of T cells affecting pathogenesis. Further investigations regarding the immunopathogenic T-cells is an important priority in the development of an effective vaccine and treatment strategies for TBE.

ACKNOWLEDGEMENTS

We thank Ikuo Takashima (Graduate School of Veterinary Medicine, Hokkaido University) for providing TBEV Oshima strain. This work was supported financially by KAKENHI [Grant-in-Aid for Challenging Exploratory Research (23658243)] from the Japan Society for the Promotion of Science, Health and Labour Sciences Research; Grant on Emerging and Re-emerging Infectious Diseases from the Japanese Ministry of Health, Labour and Welfare; the Global COE Program for Control of Emerging and Re-emerging Infectious Diseases (Nagasaki University); and the Japan Initiative for Global Research Network on Infectious Diseases.

REFERENCES

1. Dumpis U, Crook D, Oksi J. Tick-borne encephalitis. *Clin Infect Dis* 1999; 28(4): 882–890.
2. Lindquist L, Vapalahti O. Tick-borne encephalitis. *Lancet* 2008; 371(9627): 1861–1871.
3. Mansfield KL, Johnson N, Phipps LP, Stephenson JR, Fooks AR, Solomon T. Tick-borne encephalitis virus—a review of an emerging zoonosis. *J Gen Virol* 2009; 90(Pt 8): 1781–1794.
4. Gritsun TS, Lashkevich VA, Gould EA. Tick-borne encephalitis. *Antiviral Res* 2003; 57(1-2): 129–146.
5. Hayasaka D, Ivanov L, Leonova GN, Goto A, Yoshii K, Mizutani T, Kariwa H, Takashima I. Distribution and characterization of tick-borne encephalitis viruses from Siberia and far-eastern Asia. *J Gen Virol* 2001; 82(Pt 6): 1319–1328.
6. Ecker M, Allison SL, Meixner T, Heinz FX. Sequence

- analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. *J Gen Virol* 1999; 80 (Pt 1): 179–185.
7. Burke SD, Monath PT. Flaviviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, eds. *Fields virology*. Philadelphia, PA: Lippincott Williams & Wilkins; 2001. pp. 991–1041.
 8. Lundkvist K, Vene S, Golovljova I, Mavtchoutko V, Forsgren M, Kalnina V, Plyusnin A. Characterization of tick-borne encephalitis virus from Latvia: evidence for co-circulation of three distinct subtypes. *J Med Virol* 2001; 65(4): 730–735.
 9. Skarpaas T, Golovljova I, Vene S, Ljøstad U, Sjørusen H, Plyusnin A, Lundkvist A. Tickborne encephalitis virus, Norway and Denmark. *Emerg Infect Dis* 2006; 12(7): 1136–1138.
 10. Jääskeläinen AE, Tikkaoski T, Uzcátegui NY, Alekseev AN, Vaheri A, Vapalahti O. Siberian subtype tickborne encephalitis virus, Finland. *Emerg Infect Dis* 2006; 12(10): 1568–1571.
 11. Frey S, Mossbrugger I, Altantuul D, Battsetseg J, Davaadorj R, Tserennorov D, Buyanjargal T, Otgonbaatar D, Zoller L, Speck S, Wolfel R, Dobler G, Essbauer S. Isolation, preliminary characterization, and full-genome analyses of tick-borne encephalitis virus from Mongolia. *Virus Genes* 2012; 45(3): 413–425.
 12. Charrel RN, Attoui H, Butenko AM, Clegg JC, Deubel V, Frolova TV, Gould EA, Gritsun TS, Heinz FX, Labuda M, Lashkevich VA, Loktev V, Lundkvist A, Lvov DV, Mandl CW, Niedrig M, Papa A, Petrov VS, Plyusnin A, Randolph S, Suss J, Zlobin VI, de Lamballerie X. Tick-borne virus diseases of human interest in Europe. *Clin Microbiol Infect* 2004; 10(12): 1040–1055.
 13. Holzmann H. Diagnosis of tick-borne encephalitis. *Vaccine* 2003; 21 Suppl 1: S36–S40.
 14. Chiba N, Iwasaki T, Mizutani T, Kariwa H, Kurata T, Takashima I. Pathogenicity of tick-borne encephalitis virus isolated in Hokkaido, Japan in mouse model. *Vaccine* 1999; 17(7-8): 779–787.
 15. Pogodina VV, Savinov AP. Variation in the pathogenicity of viruses of the Tick-borne encephalitis complex for different animal species. I. experimental infection of mice and hamsters. *Acta Virol* 1964; 8: 424–434.
 16. Sokol F, Libikova H, Zemla J. Infectious ribonucleic acid from mouse brains infected with tick-borne encephalitis virus. *Nature* 1959; 184: 1581.
 17. Vince V, Grcevic N. Development of morphological changes in experimental tick-borne meningoencephalitis induced in white mice by different virus doses. *J Neurol Sci* 1969; 9(1): 109–130.
 18. Dorrbecker B, Dobler G, Spiegel M, Hufert FT. Tick-borne encephalitis virus and the immune response of the mammalian host. *Travel Med Infect Dis* 2010; 8(4): 213–222.
 19. Hayasaka D, Nagata N, Fujii Y, Hasegawa H, Sata T, Suzuki R, Gould EA, Takashima I, Koike S. Mortality following peripheral infection with tick-borne encephalitis virus results from a combination of central nervous system pathology, systemic inflammatory and stress responses. *Virology* 2009; 390(1): 139–150.
 20. Hayasaka D, Nagata N, Hasegawa H, Sata T, Takashima I, Koike S. Early mortality following intracerebral infection with the Oshima strain of tick-borne encephalitis virus in a mouse model. *J Vet Med Sci* 2010; 72(4): 391–396.
 21. Turtle L, Griffiths MJ, Solomon T. Encephalitis caused by flaviviruses. *QJM* 2012; 105(3): 219–223.
 22. Hayasaka D. The Development of Encephalitis Following Tick-Borne Encephalitis Virus Infection in a Mouse Model. In: Ruzek D., ed. *Daniel Ruzek*. Croatia: Intech; 2011. pp. 9.
 23. Goto A, Hayasaka D, Yoshii K, Mizutani T, Kariwa H, Takashima I. A BHK-21 cell culture-adapted tick-borne encephalitis virus mutant is attenuated for neuroinvasiveness. *Vaccine* 2003; 21(25-26): 4043–4051.
 24. Mandl CW. Steps of the tick-borne encephalitis virus replication cycle that affect neuropathogenesis. *Virus Res* 2005; 111(2): 161–174.
 25. Rumyantsev AA, Murphy BR, Pletnev AG. A tick-borne Langat virus mutant that is temperature sensitive and host range restricted in neuroblastoma cells and lacks neuroinvasiveness for immunodeficient mice. *J Virol* 2006; 80(3): 1427–1439.
 26. Mandl CW, Kroschewski H, Allison SL, Kofler R, Holzmann H, Meixner T, Heinz FX. Adaptation of tick-borne encephalitis virus to BHK-21 cells results in the formation of multiple heparan sulfate binding sites in the envelope protein and attenuation in vivo. *J Virol* 2001; 75(12): 5627–5637.
 27. Fujii Y, Kitaura K, Nakamichi K, Takasaki T, Suzuki R, Kurane I. Accumulation of T-cells with selected T-cell receptors in the brains of Japanese encephalitis virus-infected mice. *Jpn J Infect Dis* 2008; 61(1): 40–48.
 28. Hayasaka D, Aoki K, Morita K. Development of simple and rapid assay to detect viral RNA of tick-borne encephalitis virus by reverse transcription-loop-mediated isothermal amplification. *Virol J* 2013; 10: 68.
 29. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; 28(10): 2731–2739.
 30. Okonechnikov K, Golosova O, Fursov M, UGENE team. Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* 2012; 28(8): 1166–1167.
 31. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 2003; 31(13): 3497–3500.
 32. Stiasny K, Heinz FX. Flavivirus membrane fusion. *J Gen Virol* 2006; 87(Pt 10): 2755–2766.
 33. Kielian M. Class II virus membrane fusion proteins. *Virology* 2006; 344(1): 38–47.
 34. Rey FA, Heinz FX, Mandl C, Kunz C, Harrison SC. The envelope glycoprotein from tick-borne encephalitis virus

- at 2 Å resolution. *Nature Biotechnology* 1995; 375(6529): 291–298.
35. Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol* 2005; 3(1): 13–22.
 36. Zhang Y, Zhang W, Ogata S, Clements D, Strauss JH, Baker TS, Kuhn RJ, Rossmann MG. Conformational changes of the flavivirus E glycoprotein. *Structure* 2004; 12(9): 1607–1618.
 37. Stiasny K, Allison SL, Schalich J, Heinz FX. Membrane interactions of the tick-borne encephalitis virus fusion protein E at low pH. *J Virol* 2002; 76(8): 3784–3790.
 38. Fritz R, Blazevic J, Taucher C, Pangerl K, Heinz FX, Stiasny K. The unique transmembrane hairpin of flavivirus fusion protein E is essential for membrane fusion. *J Virol* 2011; 85(9): 4377–4385.
 39. Nieva JL, Agirre A. Are fusion peptides a good model to study viral cell fusion? *Biochim Biophys Acta* 2003; 1614(1): 104–115.
 40. Kroschewski H, Allison SL, Heinz FX, Mandl CW. Role of heparan sulfate for attachment and entry of tick-borne encephalitis virus. *Virology* 2003; 308(1): 92–100.
 41. Fujii Y, Hayasaka D, Kitaura K, Takasaki T, Suzuki R, Kurane I. T-cell clones expressing different T-cell receptors accumulate in the brains of dying and surviving mice after peripheral infection with far eastern strain of tick-borne encephalitis virus. *Viral Immunol* 2011; 24(4): 291–302.