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Title	Genetic and biological comparision of tick-borne encephalitis viruses from Hokkaido and Far-Eastern Russia
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Citation	Japanese Journal of Veterinary Research, 49(4), 297-307
Issue Date	2002-02-28
DOI	10.14943/jjvr.49.4.297
Doc URL	http://hdl.handle.net/2115/2927
Type	bulletin (article)
File Information	KJ00002400405.pdf

Instructions for use

Genetic and biological comparision of tick-borne encephalitis viruses from Hokkaido and Far-Eastern Russia

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Abstract

We compared the biological properties of Oshima 5-10 (tick-borne encephalitis [TBE] virus isolated in Hokkaido, Japan) and Sofjin-HO (Far-Eastern subtype TBE virus) including plaque formation, virus replication and virus protein synthesis in BHK-21 cell cultures to reveal strain differences. We also determined the complete nucleotide sequences of both strains and compared the deduced amino acid sequences. Plaques of Oshima 5-10 were smaller than those of Sofjin-HO. Virus titers in culture fluid of Oshima 5-10 were 1/100 of those of Sofjin-HO at 9 and 12 hr after infection. Less viral protein and RNA syntheses of strain Oshima 5-10 was observed than with Sofjin-HO. Genetic analysis revealed 1.4% of amino acids to differ with Sofjin-HO. No difference between the two strains was detected in the motif sequence of the viral enzyme, cleavage sites of viral protein or glycosylation sites of NS1.

Key words : Tick-borne encephalitis virus, flavivirus, genome sequence, biological property

Introduction

Tick-borne encephalitis (TBE) virus is a member of the genus *Flavivirus* in the family *Flaviviridae* and causes a fatal encephalitis in humans. TBE virus is a single-stranded positive-polarity enveloped RNA virus. The RNA genome is about 11kb in length. A single large open reading frame encodes three structural proteins (core [C], membrane[prM] and envelope [E])and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4

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B and NS5). The 5' and 3' end of the viral genome is a non-cording region (NCR)[6,29].

E protein is associated with the virus envelope and mediates both receptor binding and fusion, therefore, E protein is assumed to be an important determinant of virulence and pathogenicity[16-19,27]. NS3 protein contains motifs for serine protease[3,10,25], nucleotide triphosphatase[41], and helicase[11]. NS5 protein contains motifs for methyltransferase[24] and RNA-dependent RNA polymerase[23,32]. NS2B protein makes a complex with NS3 protein, and NS1 ,NS2A and NS4A proteins are assumed to be involved in RNA replication complex[21,22].

Based on phylogenetic analysis, TBE virus can be divided into three subtypes, Far-Eastern, European and Siberian[2,8,13,14]. The Far-Eastern subtype is known as the Russian spring summer encephalitis (RSSE) virus and prevalent in Far Eastern Russia. It is known to be highly pathogenic in humans, with a mortality rate of 5 to 20%[8].

In October 1993, a human case of TBE was reported in Kamiiso, Hokkaido, where TBE had not been reported as endemic. The virus strain, Oshima 5-10, was isolated from sentinel dogs in the same area in 1995, and identified as a TBE virus of the Far-Eastern subtype from an analysis of antigen and viral envelope(E) protein gene nucleotide sequence[38]. In Far East Russia, the annual number of TBE patients is more than 500 and fatality rates are around 15% each year[8]. In contrast, very few cases of TBE and no fatal cases had been reported in Hokkaido. Heterogenous strains with different levels of virulence are reportedly distributed in endemic areas. Our previous study showed that Oshima 5-10 strain was less virulent and replicated slower than another TBE virus, strain Sofjin-HO (the prototype of the Far-Eastern subtype) in a mouse model[7].

In this study, we compared the biological properties of the two strains including plaque formation, virus replication, and virus genome RNA and protein syntheses in BHK - 21 cell cultures to clarify strain differences. We also determined the complete nucleotide sequences of both strains and compared the deduced amino acid sequences. We discuss the correlation between biological and genetic properties.

Materials and Methods

Cells lines and viral strains

The baby hamster kidney (BHK - 21 ; ICN Biomedicals, Aurora, OH) cell line was grown at 37℃ in Eagle's minimum essential medium (MEM ; Nissui Pharmaceutical, Tokyo, Japan) containing fetal calf serum (FCS ; ICN Biomedicals). The strain of TBE virus used were Oshima 5-10 (Far-Eastern subtype TBE virus isolated from dog's blood in Hokkaido, Japan)[38], and Sofjin-HO (RSSE virus ; the prototype of the Far-Eastern subtype of TBE virus)[42]. Langat virus TP-21 strain, tick-borne flavivirus isolated from ticks in Malaysia in 1956[12] was used to obtain immune mouse ascites. The brains of virus-inoculated one-day old suckling mice were homogenized and diluted into a 1% suspension in phosphate-buffered saline (PBS ; pH7.6) containing 10% FCS and stored at - 80℃ for use as virus stock. The infectious virus titer was assayed by the focus count assay described previously[20]. Briefly, monolayers of BHK - 21 cells grown in 96 - well plates were inoculated with serially diluted virus. After 38 hours at 37℃, foci of virus on the monolayer were visualized by immunohistochemical staining by using the peroxidase-antiperoxidase procedure.

Plaque forming assay

Monolayers of BHK - 21 cells grown in 6-

well plates were infected with serially diluted virus in culture medium. After 90 minutes at 37℃, cells were washed twice in PBS and were overlaid with 2 ml of MEM containing 10% FCS and 7 % agarose (SEA KEM ME : FMC BioProducts, Rockland, ME). At 96 hr post-infection (p. i.), the cells were stained with 2 ml of MEM containing 10%FCS, 7 % agarose and 5 % neutral red. After incubation for 24 hr, the plaque morphology was observed.

Growth curve

Monolayers of BHK-21 cells grown in 24-well plates were infected with virus in culture medium at a multiiplicity of infection (m. o. i.) of 1 focus forming unit (FFU). After 90 min at 37℃, cells were washed twice with PBS and 1ml of MEM containing 4 % FCS was added. At 6 , 9 , 12, 18, 24 and 30 hr p. i., samples were harvested from the cell culture medium, stored at - 80℃ and titrated by focus count assay for quantification of the infectious virus titer.

Western blotting

Viral protein production was measured by Western blotting. Monolayers of BHK-21 cells grown in 35-mm-dishes were infected with virus in culture medium at an m. o. i. of 1 FFU. After 90 min at 37℃, cells were washed twice with PBS and 1 ml of MEM containing 4% FCS was added. At 6 , 9 and 12 hr p. i., the cells were harvested and lysed with 200 μl of sodium dodecyl sulfate (SDS) buffer (62.5 mM Tris-HCl buffer (pH 6.8), 2 % SDS, 5 % 2 - mercaptethanol, 10% glycerol and 0.005% bromo-phenol blue). After heating for 2 min at 100℃, the samples (10 μl) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For calculation of the molecular weights of proteins, a molecular standard was included in each gel.

Samples were transferred to nitrocellu-lose membranes by electroblotting, the membranes were blocked with Block Ace (Dainip-pon Pharmaceutical Co., Ltd., Osaka, Japan), and the blotted membranes were incubated with anti-Langat virus immune mouse ascites (diluted of 1 : 200) in PBS. Bound antibodies were reacted with horseradish peroxidase-conjugated anti-mouse IgG goat antibody (Zymed Laboratories, Inc., South San Francisco, CA) as a second step, and bands were visualized with 2.5 mg/ml of 4-chloro-1-naphthol (SIGMA-Aldrich Co., St Louis, MO) , 17% methanol and 0. 25% H_2O_2 in PBS.

Northern blotting

Digoxigenin (DIG) - labeled positive- or negative-sense RNA probes, encompassing the prM and E region of the Oshima - 5-10 genome, were produced by *in vitro* transcription using a DIG RNA labeling kit (Roche Diagnotics, Basel, Switzerland).

Monolayers of BHK - 21 cells grown in 35 - mm dishes were infected with virus in culture medium at an m. o. i. of 1 FFU. At 5 , 6 , 7 , 8 and 9 hr p. i., the cells were harvested and RNA was extracted using an Isogen Kit (Nippon Gene, Tokyo, Japan). The RNA samples (10 μg) were denatured with glyoxal and dimethyl sulfoxide and used in Northern blot hybridization reactions which were performed as described previously[35].

Briefly, pre-hybridization and hybridization were carried out in hybridization buffer (7 % SDS, 50 mM sodium phosphate buffer (pH7.2) , 2 % Blocking Reagent [Roche Diagnotics], 50%deionized formamide, 5×SSC(pH 7.0), 1 m M EDTA and 50 μg/ml yeast tRNA [Roche Diagnotics]) at 70℃. Both positive- and negative-sense DIG-labeled RNA probes were diluted to 0. 2 μl/ml for use.

After hybridization, the membranes were blocked with blocking buffer (1 % Blocking Reagent [Roche Diagnotics] , 0. 15 M NaCl and

0. 1 M Tris-HCl [pH 7. 5]), the bound probes were reacted with alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche Diagnotics), and the bands were visualized with 2. 5 μM CDP-Star (Roche Diagnotics) in buffer containg 0. 1 M Tris-HCl (pH 9. 5), 0. 1 M NaCl and 50 mM $MgCl_2$.

Genome sequence analysis

The nucleotide sequences of the viral genome were determined by directly sequencing the RT-PCR products. Viral RNA was extracted from brains of virus-inoculated suckling mice with the Isogen Kit. For cDNA synthesis, RT-PCR was performed with a THERMOSCRIPTTM RT-PCR System Kit (Invitrogen, Carlsbad, CA). The cycle sequencing reaction was performed with the DNA Sequence Kit (Applied Biosystems, Foster city, CA), and the sequence was determined by fluorescene autosequencer (ABI PRISM 310 Genetic Analyser, Applied Biosystems). Nucleotide sequences and deduced amino acid sequences were aligned out with GENETYX-MAC ver. 10 (Software Development, Tokyo, Japan).

Results

Plaque forming assay

To distinguish Oshima 5-10 from Sofjin-HO based on biological characteristics, we examined their plaque morphology in BHK - 21 cells (Fig. 1). BHK - 21 cells in 6 - well plates were infected with 50 FFU/well of each TBE virus and the plaques were observed at 120 hr p. i.. The plaques of Oshima 5-10 were smaller than those of Sofjin-HO.

Growth curve

To identify differences in viral replication between the two strains, the infectious virus titer of culture fluid harvested at various time points was assayed by the focus count method (Fig. 2). Virus titers in the culture fluid of Sofjin-HO increased at 9 hr p. i.. In contrast, those of Oshima 5-10 remained low until 9 hr p. i. and then increased at 12 hr p. i. to be 1/100 the level of Sofjin-HO. This result reveals that strain Oshima 5-10 replicates slower than strain Sofjin-HO.
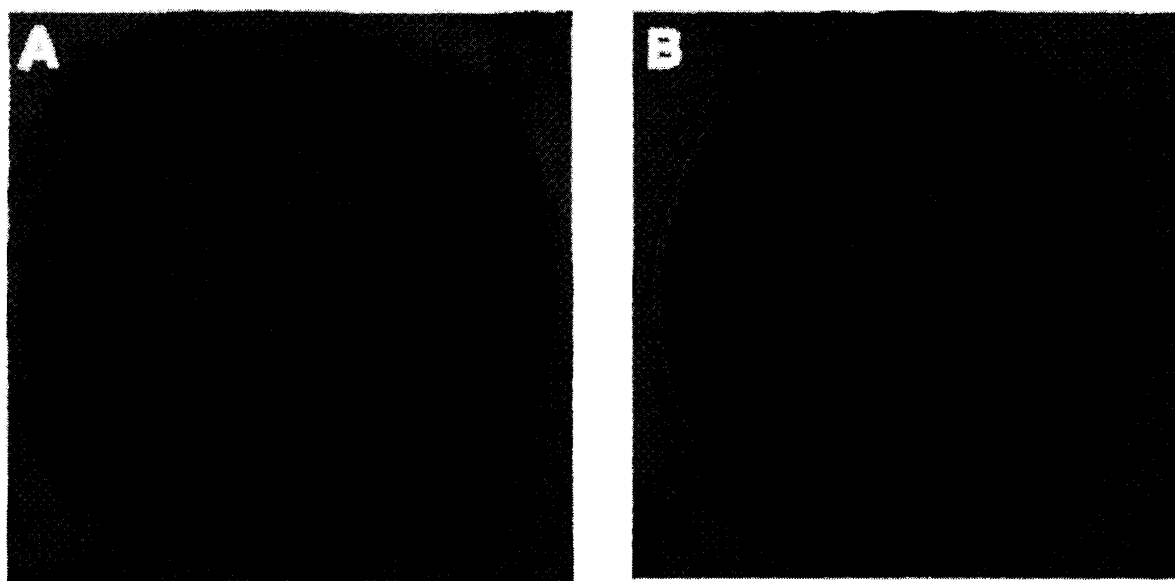

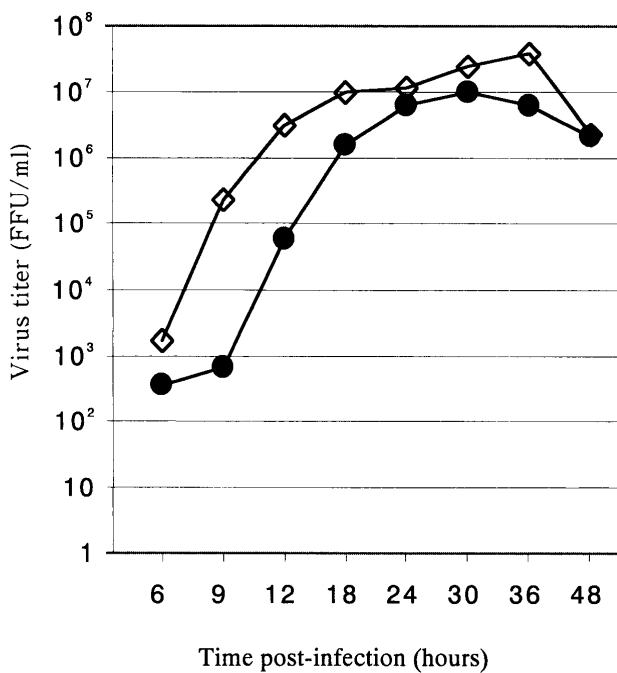Fig. 1   Morphology of plaques at 120 hr p. i.. BHK-21 cells were infected with Oshima 5-10(A) and Sofjin-HO (B).

Fig. 2. Growth curve of TBE virus. BHK-21 cells were infected at an m. o. i. of 1 FFU with either Oshima 5-10 (●) or Sofjin-HO(◇). The titer of virus released during each time period was determined by focus counting assay in BHK-21 cells.

Western blotting

Viral protein synthesis levels were compared between the two strains by Western blotting (Fig. 3). No viral protein was detected in samples from cells infected with either Oshima 5-10 or Sofjin-HO at 6 hr p. i.. At 9 hr p. i., parts of various viral proteins (E, NS1 and NS5) were detected from Sofjin-HO-infected cells, but not from Oshima 5-10-infected cells (Fig. 3). Furthermore, at 12 hr p. i., the viral proteins of Oshima 5-10 was less abundant than those of Sofjin-HO. The results show that the viral proteins of strain Oshima 5 - 10 in BHK - 21 cells were produced later than those of strain Sofjin-HO.

Northern blotting

Northern blotting analysis was carried out to compare the viral RNA synthesis of Oshima 5 - 10 and Sofjin-HO (Fig. 4). To make sure that DIG-labeled positive- or negative-sense RNA probes detect only complementary viral RNA of both senses, we carried out Northern blotting with synthesized non-labeled complementary RNA. The results showed that both probes could detect only the corresponding RNA (data not shown). We used both positive- and negative-sense RNA probes to detect viral RNA in infected BHK - 21 cells, but found no negative-strand RNA (data not shown). Positive-strand viral RNA was detected in samples from cells infected with either strain from 5 to 9 hr p. i.. Less viral RNA of Oshima 5-10 than Sofjin-HO was produced in the cells (Fig. 4).

The result shows that the RNA synthesis of Oshima 5-10 was delayed in BHK - 21 cells at an early stage, as compared with that of Sofjin-HO.

Genome sequence analysis

Nucleotide sequence analysis revealed that there were 11,100 nucleotides in the genome of Oshima 5-10 and 10,894 nucleotides in that of Sofjin-HO. This difference is caused by the shorter length of 3' NCR in Sofjin-HO as compared with Oshima 5-10[15]. On the other hands, numbers of deduced amino acid were same (3414 a. a.) in both strains. Oshima 5-10 differed by a total of 46 amino acids (1.4%) from Sofjin-HO (Table 1). No difference between the two strains was detected in the motif of the NS3 serine protease[3,10,25]/helicase[11], NS5 RNA-dependent RNA polymerase[23,32]/methyltransferase[24], cleavage sites of viral protein[1,6,9,36,37] and glycosylation sites of NS1[6].

Amino acid differences included 15 non-conservative changes which affect the characteristics of the amino acid such as polarity or hydrophobicity. NS3 protein has the most non-conservative changes (Table 1). In the 5' NCR, Oshima 5-10 and Sofjin-HO differed by
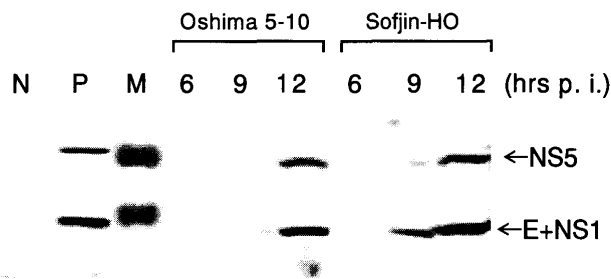
Oshima 5-10   Sofjin-HO
N P M   6   9   12   6   9   12   (hrs p. i.)

←NS5

←E+NS1

Oshima 5-10   Sofjin-HO
5 6 7 8 9   5 6 7 8 9   (hrs p. i.)

Fig. 3   Viral protein synthesis. Samples were sepa-
rated by 10% sodium dodecyl sulfate-
polyacrylamide gel electrophoresis (SDS −
PAGE). Lane N : Negative Control (BHK-
21 cells), P : Positive Control (BHK-21cells
infected with Oshima 5-10 48 hr p. i.), M :
Marker.
Samples were transferred to nitrocellulose
membranes by electroblotting, and the blot-
ted membranes were incubated with anti-
Langat virus immune mouse ascites. Bound
antibodies were reacted with horseradish
peroxidase-conjugated anti-mouse IgG goat
antibody as a second step, and bands were
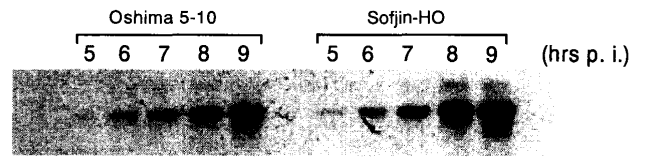visualized with 2.5 mg/ml of 4-chloro-1-
naphthol.

Fig. 4   Analysis of viral RNA replication by detec-
tion of positive-strand viral RNA.
RNA samples (10 μg) were denatured and
used in Northern blot hybridization reac-
tions which were performed as described
previously[35]. Pre-hybridization and hybridi-
zation were carried out in hybridization
buffer at 70℃. After the hybridization, the
membranes were blocked with blocking
buffer, the bound DIG-labeled probes were
reacted with alkalinphosphatase-conjugated
anti-DIG Fab fragments and bands were
visualized with CDP-Star.

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Oshima 5-10 5'      1 AGATTTTCTTGCACGTGCATGCGTTTGCTTCGGATAGCAACAGCAGCGAC    50
Sofjin-HO 5'        1 ....................G.............................    50
                      ******************* *****************************

Oshima 5-10 5'     51 AGGCTTGAGAGAGACAATCTTTTGTTTGATCAGTCGTGAACGTGTTGAGA   100
Sofjin-HO 5'       51 ...T...................CAC.........................   100
                      ***.****************...********************

Oshima 5-10 5'    101 AAAAGACAGCTTAGGAGAACAAGAGCTGGGG                      131
Sofjin-HO 5'      101 ..............................                      131
                      ******************************
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Fig. 5   Comparison of the 5' NCR of Oshima 5-10 and Sofjin-HO.

5nucleotides (Fig. 5).

Discussion

In this study, we compared the biological
and genetic characterisics of the viral strains
Oshima 5-10 and Sofjin-HO to reveal differ-
ences between TBE virus strains of the Far-
Eastern subtype.

Oshima 5-10 strain replicated more
slowly in BHK-21 cell cultures than did
Sofjin-HO. Plaques of Oshima 5-10 were also
smaller than those of Sofjin-HO (Fig. 1) and
virus titers in culture fluid were 1/100 of
those of Sofjin-HO at 9 and 12 hr after infec-
tion (Fig. 2).

Next, we examined viral protein and

Table 1 . Amino acid differences between Sofjin-HO and Oshima 5 - 10. *

Compared plotein	Amino acid diffrences (Oshima 5-10→Sofji-HO)	Total	Percentage idenity
C	Arg-32→Gln* Met-43→Leu Ala-54→Val Asn-64→Lys* Val-111→Leu	5	95.5
prM	Ile-134→Met Val-145→Ala	2	98.8
E	Ala-153→Val Val-308→Met Met-462→Val Ala-463→Val	4	99.2
NS1	Leu-58→Val Val-72→Ala Phe-93→Leu Ile-138→Thr* Val-346→Ile	5	98.6
NS2A	Arg-52→Lys Ile-168→Thr* Ala-169→Val Val-225→Ile	4	98.3
NS2B		0	100
NS3	Asp-2→Gly* Phe-45→Ser* Ser-62→Tyr Val-63→Ile Val-68→Ala Gly-124→Arg* Ile-184→Thr* Thr-380→Ala * Gly-85→Ser *	9	98.6
NS4A	Ala-63→Gly	1	99.3
NS4B	Leu-24→Val Phe-88→Leu Ser-198→Ala * Ser-227→Gly *	4	98.4
NS5	Lys-15→Arg Arg-101→Lys Met-130→Val Tyr-519→His * Thr-634→Ser Phe-642→Leu Arg-677→Gly * Val-692→Ile Phe-778→Leu Gly-827→Ser* Val-831→Ala Arg-862→Lys	12	98.7
polyprotein		46	98.6

*The sequences used in this analysis were as follows (GenBank numbers in parentheses) : Oshima 5-10 (AB 062063), Sofjin-HO (AB062064).

RNA levels of both strains early in the infection when the difference was most evident. Protein production was delayed in Oshima 5-10 infected cells as compared with in Sofjin-HO infected cells(Fig. 3). Viral RNA was also detected later in strain Oshima 5-10 than Sofjin-HO (Fig. 4) . The later onset of protein and RNA production in Oshima 5-10 may be related with the slower viral replication in BHK - 21 cells.

We also determined the complete nucleotide sequences of both strains and compared their deduced amino acid sequences. There were 5 nucleotides differences in the 5' NCR gene between Oshima 5-10 and Sofjin-HO (Fig. 5). There are reports that attenuation of flavivirus was caused by changes of nucleotides in the 5' or 3' NCR[5, 28, 31,33]. The 5' NCR is capped[6] and the 5' and 3' NCR make secondary structure and have the inverted complementary repeat sequences which are considered to play an important role in viral transcription[13]. But in this study, there was no difference in those regions between the two strains and we could not conclude that above mentioned 5 nucleotide differences had influence on virus replication.

Oshima 5-10 possesed a total of 46 amino acids (1.4%) that differed from Sofjin-HO(Table1). There are reports that attenuation of flavivirus was caused by changes of amino acids in the motif sequence of the NS proteins[4,27,40], cleavage sites of viral protein[1,9,16,36] and glycosylation sites of NS1[30]. Particularly, a lack of motif sequence of NS3 serine protease[3,10,25] / helicase[11] , NS 5 RNA-dependent RNA polymerase[23,32]/methyltransferase[24] is lethal. But in this study, there was no difference in those regions between the two strains.

The 46 amino acid changes included 15 non-conservative types. In flavivirus, E protein is assumed to be an important determinant of virulence and pathogenicity, because

it is associated with the viral envelope and mediates both receptor binding and fusion[16-19,27,29,34]. However, sequence analyses of the E protein gene of Oshima 5-10 showed high identity with Sofjin-HO and the amino acid differences between the two strains did not include non-conservative changes[15]. Therefore, other proteins may influence the biological characteristics and virulence. But we could not correlate these amino acid differences with the biological differences between the strains.

We demonstrated that in BHK - 21 cells, Oshima 5-10 replicated at a more slowly pace and produced protein and RNA later than Sofjin-HO. The results encourage further study to correlate the biological, genetic and pathogenetic characteristics of the two strains for understanding the virulence of the TBE virus.

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