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Title: Characterization of Tick-borne Encephalitis Virus Isolated From a Tick in Central Hokkaido in 2017

Author: Yuji Takahashi [1)], Shintaro Kobayashi [1)], Mariko Ishizuka [1)], Minato Hirano [1)], Memi Muto [1)], Syoko Nishiyama [2)], Hiroaki Kariwa [1)], Kentaro Yoshii [1),3)]

Affiliation:

1) Laboratory of Public Health, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan
2) Laboratory for zoonotic disease, Joint department of veterinary Medicine, Faculty of applied biological sciences, Gifu University
3) National Research Center for the Control and Prevention of Infectious Diseases (CCPID), Nagasaki University

Corresponding author: Kentaro Yoshii,

Postal address: National Research Center for the Control and Prevention of Infectious Diseases (CCPID), Nagasaki University, 1-12-4 Sakamoto, Nagasaki, 852-8523, Japan

Tel/fax: +81-95-819-8595

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

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Summary

Tick-borne encephalitis virus (TBEV) is a zoonotic virus in the genus *Flavivirus*, and family *Flaviviridae*. TBEV is widely distributed in northern regions of the Eurasian continent, including Japan, and causes severe encephalitis in humans. Tick-borne encephalitis (TBE) was recently reported in central Hokkaido, and wild animals with anti-TBEV antibodies were detected over a wide area of Hokkaido, although TBEV was isolated only in southern Hokkaido. In this study, we conducted a survey of ticks to isolate TBEV in central Hokkaido. One strain, designated Sapporo-17-Io1, was isolated from ticks (*Ixodes ovatus*) collected in Sapporo city. Sequence analysis revealed that the isolated strain belonged to the Far Eastern subtype of TBEV and was classified in a different subcluster from Oshima 5-10, which was isolated in southern Hokkaido previously. Sapporo-17-Io1 showed similar growth properties to those of Oshima 5-10 in cultured cells and mouse brains. The mortality rate of mice infected intracerebrally with each virus was similar, but the survival time of mice inoculated with Sapporo-17-Io1 was significantly longer than that of mice inoculated with Oshima 5-10. These results indicate that the neurovirulence of Sapporo-17-Io1 was lower than that of Oshima 5-10. Using an infectious cDNA clone, replacement of genes encoding non-structural genes from Oshima 5-10 with those from Sapporo-17-Io1 attenuated the neuropathogenicity of the cloned viruses. This result indicated that the non-structural proteins determine the neurovirulence of these two strains. Our results provide important insights for evaluating epidemiological risk in TBE endemic areas of Hokkaido.

Introduction

Tick-borne encephalitis virus (TBEV), a member of the genus *Flavivirus* within the family *Flaviviridae*, causes severe encephalitis in humans, referred to as tick-borne encephalitis (TBE). TBEV is prevalent throughout a wide area of northern Eurasia, including Europe, Russia, Far Eastern Asia, and Japan (1, 2) More than 10,000 patients with TBE are reported annually. Based on phylogenetic analysis, TBEV can be divided into three subtypes: European, Siberian, and Far Eastern (3, 4), and Baikalian and Himalayan subtypes were also recognized recently (5-7). These three subtypes cause different symptoms and have different mortality rate. The European subtype, which is distributed throughout Europe, causes a biphasic fever and a relatively mild form of encephalitis, and its mortality rate is up to 2% (8, 9). The Siberian subtype, which is widely distributed in Russia, causes a relatively mild form of encephalitis, and the case mortality rate is 6-8%. In some reports, humans infected with the Siberian subtype develop chronic disease (1). The distribution range of the Far Eastern subtype covers eastern Russia, northern China, and northern Japan. Infection with this subtype of TBEV causes the most severe neural disorders, including encephalitis and meningoencephalitis, and has a mortality rate up to 30% (3, 4).

TBEV is maintained mainly in ticks of the genus *Ixodes* and wild vertebrate hosts especially rodents, such as *Apodemus*, *Myodes*, and *Microtus* species in nature (10, 11). Each subtype of TBEV is transmitted by different vectors, including *Ixodes ricinus* and *I. persulcatus* in the European and Siberian areas, and primarily by *I. persulcatus* in the far eastern area (3), but transmissions by other tick species were also reported. Humans are not involved in the natural transmission of TBEV and are only accidental hosts due to tick bites.

In 1993, the first confirmed case, which was serologically diagnosed as TBE, was reported in the southern portion of Hokkaido, the northern island of Japan (2). TBEV was isolated from dogs, ticks (*I. ovatus*), and rodents (*Apodemus speciosus*) in that area (11-13), and the virus was identified as the Far Eastern subtype of TBEV through phylogenetic analysis. TBEV was isolated only in southern

Hokkaido, although our epizootiological survey indicated that TBEV was present in wild animals in Hokkaido and other areas of Japan (14-16). Four more cases of TBE were reported throughout Hokkaido, including in the central region between 2016 and 2018 (16-19). In our seroepidemiological studies in humans, a meningoencephalitis patient suspected of having Lyme disease was found to be infected with TBEV, and unrecognized subclinical infections of TBEV were detected among members of the Japan Self-Defense Forces (20, 21). Therefore, it is necessary to isolate and characterize TBEV from wide areas of Hokkaido to evaluate of the epidemiological risk of TBE.

In this study, we collected ticks in central Hokkaido and attempted to isolate TBEV. We isolated TBEV from cell cultures inoculated with a tick homogenate. The isolated TBEV was analyzed phylogenetically by sequencing of genomic RNA, and the growth properties and pathogenicity of the isolated virus were examined in cell cultures and a mouse model.

Methods

Tick collection and virus isolation

Ticks were collected from three areas in central Hokkaido in 2017: a mountain forest in southwestern Sapporo city; Nopporo Forest, which straddles the cities of Sapporo, Ebetsu, and Kita-Hiroshima; and Maoi Hills, located between the town of Naganuma and Yuni (Fig. 1). In total, 1,923 ticks were collected by dragging flannel sheets over the vegetation and pooled into groups of 10–15 ticks. The ticks were homogenized in phosphate buffered saline (PBS) using a pestle. Each homogenized suspension was centrifuged, and the supernatant was collected and stored at −80 °C until use.

Baby hamster kidney-21 (BHK) cells grown in 24-well plates were inoculated with the tick homogenates, and incubated at 37 ◦C under 5% CO_2. After 2–4 days, the cells were inspected for cytopathic effects (CPEs) and the supernatants of cells showing CPE were harvested and stored at −80 °C. The viruses in these samples were identified by reverse-transcription polymerase chain reaction (RT-PCR) and immunofluorescence assay (IFA). Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking with 2% bovine serum albumin, the cells were incubated with polyclonal hyperimmune murine ascites fluid from mice infected with Langat virus which is cross-reactive with TBEV, followed by Alexa 555-conjugated anti-mouse immunoglobulin G antibodies (Thermo Fisher Scientific, Waltham, MA). Viral RNA was extracted from BHK cells using ISOGEN II (Nippon Gene, Tokyo, Japan) and reverse-transcribed using random primers and Superscript III reverse-transcriptase (Thermo Fisher Scientific). TBEV-specific sequences were amplified using Platinum Taq polymerase (Thermo Fisher Scientific). To amplify the envelope (E) protein gene of TBEV, we used the following primers specific for Far Eastern TBEV: (forward) 5'-AGATTTTCTTGCACGTGCAT-3' and (reverse) 5'-GCACACTGTGTATGTAAGAC-3'. All stock viruses were propagated once in BHK cells. The TBEV strain isolated in this study was designated Sapporo-17-Io1 (Genbank accession number LC440459).

110 *TBEV gene sequencing and phylogenetic analysis*

111 The nucleic acid sequences of viral genomes were determined by direct sequencing. Cycle sequencing
112 reactions were performed using the BigDye^{TM} Terminator Cycle Sequencing Kit (Thermo Fisher
113 Scientific), and the sequences were determined using the 3130 Genetic Analyzer (Thermo Fisher
114 Scientific). Phylogenetic analysis was performed using the complete genomic sequences of TBEV
115 strains. Genetyx version 8 was used for multiple sequence alignment. MEGA X
116 (http://www.megasoftware.net/) was used to generate phylogenetic trees using the neighbor-joining
117 method. The reliability of the dendrogram was evaluated using 500 bootstrap replicates.

118

119 *Virus titration and growth curves in cell culture*

120 The BHK cell line was grown at 37 °C in Eagle's minimum essential medium (FUJIFILM Wako Pure
121 Chemical Corporation, Osaka, Japan) supplemented with 8% fetal calf serum (FCS; BioWest, Nuaillé,
122 France). The SH-SY5Y cell line, derived from a human neuroblastoma, was grown at 37 °C in
123 Dulbecco's modified Eagle's medium (FUJIFILM Wako Pure Chemical Corporation) supplemented
124 with 10% FCS.

125 The TBEV Oshima 5-10 strain was isolated from a dog in 1995 in Hokuto city (2). After two passages
126 in suckling mouse brain, BHK cells were infected with the virus. The culture supernatant was collected
127 on days 3 to 5 and frozen at –80 °C until use.

128 For titration, BHK cell monolayers prepared in 12-well plates were incubated with serial dilutions of
129 viral suspension for 1 h, overlaid with minimal essential medium containing 2% FCS and 1.5%
130 carboxymethyl cellulose, and then incubated for 4 days. The cells were fixed and stained with crystal
131 violet (0.25% in 10% buffered formalin) to visualize plaques. Finally, plaques were counted, and viral
132 titers are presented as plaque-forming units (pfu).

133 For growth curve experiments, subconfluent BHK and SH-SY5Y cells were grown in 12-well plates
134 and then inoculated with the virus at a multiplicity of infection of 0.01 pfu ml^{-1}. The cells were
135 incubated at 37 °C under 5% CO_2. Supernatant samples were harvested at 12, 24, 48, and 72 h post-

infection and stored at −80 °C until titration.

Animal model

Each virus was inoculated subcutaneously with 10e3 pfu and intracerebrally with 50 pfu into 10 female BALB/c mice (5 weeks old; Japan SLC, Shizuoka, Japan). Surviving mice were monitored for 28 days post-infection (d.p.i.) to obtain survival curves and mortality rates. The onset of disease was indicated by a 10% weight loss compared with the weight prior to viral infection. For analysis of the viral distribution among tissues, three to six mice were sacrificed at 3, 6, and 9 d.p.i. to collect the brains. The brains were individually weighed, homogenized, and prepared as 10% (w/v) suspensions in PBS. The suspensions were clarified via centrifugation (5,000 rpm for 5 min at 4 °C), and the supernatants were titrated. All 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. All experimental protocols were approved by the Animal Care and Use Committee of Hokkaido University (18-0027).

Histopathological examination

Mice that had been infected intracerebrally with 50 pfu TBEV were sacrificed at 3, 6, and 9 d.p.i. The brains of these mice were collected in formalin for fixation, and the fixed brain tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, as described previously (22). Immunohistochemical detection of TBEV antigens was performed using rabbit polyclonal antibodies against the E protein (23), and the sections were stained using the streptavidin–biotin–immunoperoxidase complex method (Histofine SAB-PO kit: Nichirei, Tokyo, Japan). The sections were counterstained with Mayer’s hematoxylin.

Plasmid preparation

Sapporo-REP. Total cellular RNA was extracted from BHK cells infected with the Sapporo-17-Io1 strain as described previously. Three fragments of Sapporo-17-Io1 cDNA were amplified using PrimeSTAR GXL DNA polymerase (Takara Bio Inc.). Primers were designed based on the nucleotide (nt) sequence of Sapporo-17-Io1. The first fragment (nt 1–238) contained an SP6 promoter recognition site preceding the first base of the viral genome. The first fragment was designed to fuse to the fragment of the capsid (C) protein gene in-frame to the C-terminal fragment of the E protein gene, serving as a signal sequence for the non-structural protein 1 (NS1). A synthetic oligonucleotide was used to add *Avr* II sites at nt positions 238–243 and 2291–2296. This silent mutation was engineered to permit ligation of the second fragment (nt 2294–3965) and translation of the signal sequence for NS1. The third and fourth fragments were located at nt 3966–5514 and nt 5515–9216, respectively. The last fragment (nt 9217–11111) contained a *Spe* I restriction endonuclease site. The fragments were resolved by gel electrophoresis and purified using Wizard® SV Gel and PCR Clean-Up system (Promega, Madison, USA). The fragments were digested with restriction endonucleases and ligated into the low copy plasmid pGGVS209 (24) (Fig. 2).

Sapporo-IC. The full-length infectious cDNA clone, Sapporo-IC, was created by inserting the coding regions of the structural protein genes into the Sapporo-REP plasmid. Fragments containing C, prM, and E (nt 239-2293) were amplified using PrimeSTAR GXL DNA polymerase (Takara Bio Inc.) from the parent Sapporo-17-Io1 RNA. The sense primer included an *Avr* II restriction endonuclease site. The PCR products were digested with *Avr* II and inserted into the Sapporo-REP plasmid, which was also digested with *Avr* II (Fig. 2).

Oshima-IC, and Oshima-REP. Oshima-IC, encoding the full-length cDNA of the TBEV Oshima 5-10 strain (AB062063), was prepared as described previously (25). The Oshima-REP plasmid was used for preparation of replicon RNAs of the Oshima 5-10 strain, as described previously (25)

Chimeric virus of Sapporo-17-Io1 and Oshima 5-10. Oshima-IC/Sapporo-CME and Sapporo-IC/Oshima-CME were constructed by replacing the structural protein genes with those of the Sapporo-17-Io1 and Oshima 5-10 strains in the Oshima-IC and Sapporo-IC plasmids, respectively. PCR fragments for each structural protein gene were amplified, and inserted into the Oshima-REP and Sapporo-REP plasmids, as described above.

mRNA transcription and transfection

Replicon or infectious clone plasmids were linearized using *Spe* I and transcribed into RNA using the mMESSAGE mMACHINE SP6 Kit (Ambion). The mRNA samples were treated with DNase I and precipitated with LiCl. The precipitated RNA was dissolved in 30 μL DEPC-treated water. BHK cells were transfected with the mRNA using Lipofectamine MessengerMAX reagent (Thermo Fisher Scientific). Recombinant viruses from the mRNA were collected from the supernatant at 3–4 days post-transfection, and the working stocks of the Sapporo-IC and Oshima-IC viruses were propagated in BHK cells, as described above.

Statistical analysis

Data are expressed as means ± the standard errors. The Student's t-test was used to determine statistical significance of the differences in the survival time and virus titers at each time point. The Kaplan-Meier survival curves and the log-rank test were used to evaluate the survival curves of infected mice.

Result

Isolation and identification of TBEV in Sapporo

Ticks were collected from the study area where wild animals positive for anti-TBEV antibodies were detected in previous study (unpublished data). In total, 1,923 ticks were collected via flagging in these areas. *I. ovatus* was the predominant tick species, and small numbers of *I. persulcatus*, and *Haemaphysalis flava* were also collected (Table 1).

All collected ticks were pooled by 10 ticks for same species. BHK cells were inoculated with each homogenate of the tick pools and the supernatants were passaged once. After 3-4 days of incubation, CPEs were observed in cells inoculated with one homogenate of *I. ovatus.* Virus-specific antigens and bands were detected in the cells by IFA and RT-PCR, respectively (Fig. 3). This isolate was identified as TBEV and designated Sapporo-17-Io1.

Genetic analysis of the isolated TBEV strain

The complete genomic sequence of Sapporo-17-Io1 was determined. A phylogenetic tree of the complete genome is shown in Fig. 4. Sapporo-17-Io1 was classified as the Far Eastern subtype of TBEV, belonging to a different cluster than the Oshima 5-10 strain.

The nt and amino acid (aa) sequences of the complete genomes of Sapporo-17-Io1 and Oshima 5-10 were compared (Supplementary Table 1 and 2). The nt and aa homologies were 95.9% (10645/11101 nt) and 98.9% (3377/3415 aa), respectively, in complete sequences including the 5’ and 3’ untranslated regions (UTRs). A 3-nt deletion and 1-nt insertion were observed in the 3’ UTR of Sapporo-17-Io1 compared with the 3’ UTR of Oshima 5-10. The nt and aa homologies of each gene were 95.1–97.2%, and 96.9–100%, respectively, and no deviation was observed in the differences among genes.

233 *Growth properties and pathogenicity of Sapporo-17-Io1*

234

235 The growth properties of Sapporo-17-Io1 were compared with those of Oshima 5-10 by monitoring
236 viral replication after infection. BHK and SH-SY5Y cells were infected with each virus at a
237 multiplicity of infection of 0.01. Viruses were harvested at 24, 48, and 72 h post-infection and titers
238 were measured using a plaque assay. The viral growth titers in BHK and SH-SY5Y cells were almost
239 identical between Sapporo-17-Io1 and Oshima 5-10 (Fig. 5A and B).
240 The pathogenicity of Sapporo-17-Io1 was also compared with that of Oshima 5-10 in a mouse model.
241 Each mouse was infected subcutaneously with 10^3 pfu or intracerebrally with 50 pfu of each viral
242 strain and their survival rates were recorded over 21 days (Fig. 6A and B).
243 After subcutaneous inoculation, 20% of mice infected with Sapporo-17-Io1 and 80% of mice infected
244 with Oshima 5-10 showed mild symptoms, such as a hunched posture, weight loss, and ruffled fur, or
245 severe symptoms, such as paralysis and loss of balance. The mortality rate was 20% in mice infected
246 with Sapporo-17-Io1 and 40% in mice infected with Oshima 5-10. On the other hand, in the mice with
247 intracerebral inoculation, almost all mice infected with either strain showed severe neurological
248 symptoms and died. Although the survival curves were not different significantly, the survival time
249 was significantly longer in mice infected with Sapporo-17-Io1 than in those infected with Oshima 5-
250 10 ($P < 0.05$) (Fig. 6D). Differences of clinical symptoms were not observed between the mice infected
251 with the two strains.
252 To examine viral replication in the brain, 50 pfu/mouse of Sapporo-17-Io1 or Oshima 5-10 was
253 inoculated into mice intracerebrally. Viral multiplication in the brain did not differ substantially
254 between Sapporo-17-Io1 and Oshima 5-10 on 3, 6, or 9 d.p.i (Fig. 6C).

255

256 *Histopathology of Sapporo-17-Io1*

257

Histopathological analysis was conducted on the brains of mice inoculated intracerebrally with Sapporo-17-Io1 or Oshima 5-10, at 6, and 9 d.p.i. No notable histopathological changes, such as neuronal degeneration, perivascular infiltration of inflammatory cells, or glial cell proliferation, were observed after either Sapporo-17-Io1 or Oshima 5-10 infection (Fig. 7).

Viral antigens were detected diffusely in the cytoplasm of neurons located mainly in the cerebral cortex and thalamus mainly, but also faintly in the medulla of mice infected with Sapporo-17-Io1 or Oshima 5-10 (Fig. 7). The distribution of viral antigens was similar between the two strains, but the level of antigens tended to be lower for Oshima 5-10 than Sapporo-17-Io1.

Pathogenicity of recombinant viruses from Sapporo-17-Io1 and Oshima 5-10

We constructed an infectious cDNA clone of Sapporo-17-Io1 (Fig. 2) for further analysis. Its growth properties in BHK cells were almost similar to those of Sapporo-IC and Oshima-IC (data not shown). Each mouse was inoculated intracerebrally with 50 pfu Sapporo-IC or Oshima-IC. As observed in mice infected with wild-type Sapporo-17-Io1 and Oshima 5-10, almost all mice died and the survival curves were not different significantly, with a significantly longer survival of mice infected with Sapporo-IC compared with Oshima-IC ($P < 0.05$) (Fig. 8B,C).

To investigate the viral factors responsible for the difference in pathogenicity between the two strains, we constructed two chimeric viruses by replacing the structural protein genes in one strain with those in the other strain (Sapporo-IC/Oshima-CME and Oshima-IC/Sapporo-CME) (Fig. 8A). The mortality rate of mice infected with each chimeric virus was also 90%. The survival time of mice infected with Oshima-IC was similar to that of mice infected with Oshima-IC/Sapporo-CME but significantly different from that of mice infected with Sapporo-IC/Oshima-CME ($P < 0.05$) (Fig. 8B,C). These results indicated that NS proteins of Sapporo-17-Io1 are responsible for the differences in pathogenicity.

Discussion

In this study, a new strain of TBEV belonging to the Far Eastern subtype was isolated from *I. ovatus* collected in Sapporo city, central Hokkaido, Japan (Figs. 1, 3 and 4). All ticks were collected from mountain forest areas near Sapporo city, and ticks infected with TBEV were collected from a mountain site in Sapporo city (Fig. 1). Sapporo is the prefectural capital of Hokkaido, and its western and southern suburbs are surrounded by mountains covered with deep forests. Wild animals including deer, raccoons, and rodents that harbor ticks are also widely distributed in the area (11, 12, 14, 16), and thus the natural environment has supported the transmission cycle of TBEV. Some animals in Hokkaido were found to possess anti-TBEV antibodies, and TBEV was detected in dogs, rodents, and ticks in our previous studies (2, 11-13). Furthermore, five people were infected with TBEV via tick bites in the southern, central, and northern areas of Hokkaido (2, 17-19). These findings indicate that TBEV is already widespread in wild animals and ticks throughout Hokkaido and are supported by the recent case reports.

In this study, TBEV was isolated from *I. ovatus*, from which Oshima 5-10 was isolated in a previous study (12). *I. ovatus* is the predominant tick species throughout Japan including Hokkaido. *I. persulcatus*, which is a major TBEV vector on the Eurasian continent, is also distributed widely in Japan, especially in northeastern Hokkaido, but is a minor species in Japan (26). TBEV may become endemic in Japan with transmission by *I. ovatus*, as TBEV occasionally changes tick vectors based on the tick fauna of each region. For example, in South Korea, *Haemaphysalis longicornis* and *H. flava* transmit the European subtype of TBEV, which is transmitted mainly by *I. ricinus* in Europe, as the predominant ticks in South Korea were *Haemaphysalis spp.* (27-32). The minimum field detection rates of TBEV in *I. ovatus* were 0.05% (1/1839) in this investigation and 0.33% (2/600) in our previous study in Southern Hokkaido. These rates are lower than those of the Far Eastern and Siberian subtypes of TBEV in *I. persulcatus* in far eastern Russia and Mongolia (1–9%) (33-36). However, given that the isolation rates of TBEV from rodents (1.2% and 2.9%) and the proportion of seropositive rodents

309 in Hokkaido (12.4% and 10.4%) (11, 13) were similar to those of TBEV in other endemic areas of the
310 continent (10), the low detection rate in *I. ovatus* found in this study likely did not affect the circulation
311 of TBEV between ticks and rodents in Hokkaido. It is also possible that the differences might be
312 attributable to each sampling scale, and further study is required to elucidate it.
313 The phylogenetic analysis showed that Sapporo-17-Io1 belongs to a different subcluster of the Far
314 Eastern subtype from that of Oshima 5-10 (Fig. 4). As these subclusters diverged several hundred years
315 ago, it is likely that these Japanese strains of TBEV arrived in Hokkaido independently over the past
316 few centuries (33). Because Hokkaido is 1,000 km from the Eurasian continent across the Japan sea,
317 the invasion of TBEV was likely via ticks adhered to birds (37) traveling to Hokkaido. Because many
318 birds migrate from far-eastern Russia to Japan by way of Hokkaido, TBEV may have already been
319 established not only on Hokkaido but over wide areas of Japan (38, 39).
320
321 The strain isolated in this study, Sapporo-17-Io1, was less virulent in the mouse model than was
322 Oshima 5-10 (Fig. 6B). The multiplication of Sapporo-17-Io1 was similar to that of Oshima 5-10, and
323 the mortality rate and average survival time of mice infected subcutaneously with Sapporo-17 Io1 were
324 the same as those of mice infected with Oshima 5-10 (Fig. 5 and 6A, C). However, the survival time
325 of mice infected intracerebrally with Sapporo-17-Io1 was significantly longer than that of mice
326 infected with Oshima 5-10. In addition, although the levels of viral antigens in the central nervous
327 system (CNS) tended to be lower after Oshima 5-10 infection than after Sappporo-17-Io1 (Fig. 7),
328 Oshima 5-10 caused more severe pathogenicity in mice compared with Sapporo-17-Io1, as well as
329 other strains of TBEV (40, 41). These results suggest that the difference in pathogenicity between these
330 two strains was not due directly to viral multiplication in the CNS, but rather to functional alternations
331 causing damage to the CNS such as neuronal dysfunction and degeneration.
332 Neurological diseases have been associated with neuronal dysfunction and degeneration caused by
333 infection with neurotropic viruses. Rabies infection affects ion channels and neurotransmission
334 resulting in functional impairment (42). Alterations in synaptic function have also been reported with

335 Borna disease viral infection (43, 44). Axonal degeneration is instrumental to the development of
336 neuronal dysfunction during herpes virus and human immunodeficiency virus (HIV) infections (45-
337 48). TBEV infection caused neuronal dysfunction by regulating the transport of dendritic mRNA via
338 the 5' UTR of the viral genomic RNA (49) and also suppressed neurite outgrowth, which is involved
339 in the development of neurological diseases (50). As described above, neurotropic viruses including
340 TBEV cause neuronal dysfunction during neuropathogenesis. It is possible that the genetic differences
341 between Sapporo-17-Io1 and Oshima 5-10 affect this role.
342
343 Recombinant viruses recovered from infectious cDNA clones were analyzed to identify the viral
344 genetic factors driving the difference in pathogenicity (Fig. 8). Viral quasispecies from naturally
345 isolated strains, including TBEV, have been reported to affect pathogenicity (51-54). The viruses
346 cloned from parental Sappporo-17-Io1 and Oshima 5-10 showed differences in pathogenicity, as
347 observed in the wild-type strains (Fig. 8B). These results indicate that the viral genetic factors of the
348 two strains affected their difference in pathogenicity in addition to the effect on quasispecies.
349 Replacement of the NS protein genes of Oshima 5-10 with those of Sapporo-17-Io1 attenuated the
350 pathogenicity of the cloned viruses (Fig. 8B,C). The two strains had 28 aa differences in their NS
351 proteins (Supplementary Table 1 and 2). In addition to the roles in viral genome replication and virion
352 assembly, NS proteins can modulate host responses via interactions with host factors (55-58). Several
353 NS proteins have been shown to affect innate immunity mechanisms in the host, such as toll-like
354 recepter signaling and interferon responses (59-64), which suppress the neuropathogenicity of
355 neurotropic flavivirus (57, 58, 65, 66). Furthermore, NS5 induced defects in neuronal development via
356 interactions with host factors involved in the construction of neuronal circuits, such as Scribble and
357 Rac1 (56, 67, 68). Several aa of the NS5 protein of TBEV are involved in the attenuation of neurite
358 outgrowth (50). These alterations of the host response by NS proteins were assumed to cause the
359 development of neurological disease during neurotropic flaviviral infection. However, the differing aa
360 in NS proteins observed in Sapporo-17-Io1 and Oshima 5-10 have not been reported to be involved in

virulence in mice. Identification of the viral factors responsible for the difference in virulence between these two strains will further our understanding of the functions of these viral proteins in the pathogenicity of the Far Eastern subtype of TBEV.

In summary, we isolated the Far Eastern subtype of TBEV from *I. ovatus* in central Hokkaido, where cases of TBE have been reported recently. The isolated strain, Sapporo-17-Io1, was classified in a different subcluster from the other isolate collected in southern Hokkaido, indicating independent invasion by these two strains into Hokkaido. Moreover, the two strains showed different neurovirulence in a mouse model, indicating that natural mutations between the two strains especially in NS protein genes, affect pathogenicity. Further investigation is required to determine the detailed distribution of TBEV in other regions of Japan, and evaluating the nationwide epidemiological risk of neuropathogenicity due to TBEV will clarify the pathogenic mechanism of TBEV, supporting the development of antiviral treatments.

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Author contributions

Y. T. – conceptualization, methodology, investigation, original draft preparation.

S.K. – conceptualization, methodology, review and editing

M. I. – methodology, investigation.

M. H. – methodology, investigation.

M. M. – methodology, investigation.

S. N. – methodology, investigation.

H. K. – conceptualization, review and editing.

K. Y. – conceptualization, methodology, investigation, review and editing.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Table 1 Numbers of ticks collected in the case study area by flagging.

Species	Stage	Sex	Sapporo city	Nopporo area	Maoi hill
Ixodes ovatus	Adult	Male	361	94	372
		Female	404	147	452
	Nymph		9	0	0
Ixodes persulcatus	Adult	Male	25	4	3
		Female	11	4	2
Haemoaphysalis flava	Adult	Male	2	1	1
		Female	5	2	2
	Nymph		12	4	6

561

562 **Figure Legends**

563

564 **Fig. 1.** Geographical locations of the study sites. A mountain forest area in Sapporo, Nopporo
565 Forest, and Maoi Hills are indeicated by (a), (b), and (c), respectively.

566

567 **Fig. 2.** Schematic representation of the TBEV genome, and construction of the replicon and
568 infectious cDNA clone of TBEV Sapporo-17-Io1. (a) Five fragments were cloned into the low-copy
569 plasmid $pGGV_{s209}$. The Sapporo replicon regions were inserted under the control of the SP6
570 promoter. An *Spe* I restriction endonuclease site was fused to the 3' end of the viral genome. (b) To
571 construct the infectious cDNA clone, the coding regions of the C, prM, and E genes were inserted
572 into the Sapporo-REP plasmid.

573

574 **Fig. 3.** Detection of TBEV specific antigen and RNA. BHK cells inoculated with the supernatant
575 from TBEV positive tick homogenates (Sapporo-17-Io1) (A) and mock-infected cells (B). Cells were
576 stained with anti-tick-borne flavivirus antibodies. (C) The sequence from the 5' UTR to E protein of
577 TBEV (1889 bp), collected from cells inoculated with TBEV positive tick homogenate (Saporo-17-
578 Io1), was amplified by RT-PCR. Cells infected with Oshima 5-10 and mock treated cells were used
579 as controls.

580

581 **Fig. 4.** Phylogenetic tree of TBEV strains based on complete viral genomes. The arrow indicates
582 TBEV Sapporo-17-Io1 isolated in this study, and the arrowheads indicate TBEV Oshima strains
583 isolated from southern Hokkaido in previous reserch. The GenBank accession numbers of the viruses
584 used in this study are shown after each strain name.

585

586 **Fig. 5.** Comparison of the growth curves of Sapporo-17-Io1 and Oshima 5-10 in BHK cells (A)
587 and SH-SY5Y cells (B). A monolayer of each cell type was infected with each virus at a multiplicity
588 of infection of 0.01. At each time point, the medium was harvested and viral titers in BHK cells were
589 determined using a plaque assay. The virus detection limit for the assay was 10^2 pfu ml^{-1}. Error bars
590 represent standard deviations.

591

592 **Fig. 6.** Survival of mice inoculated with Sapporo-17-Io1 and Oshima 5-10. Mice were inoculated
593 subcutaneously with 10^3 pfu (n = 10) (A) or intracerebrally with 50 pfu (n = 15) (B) of each virus
594 and monitored for 28 days. (C) Viral multiplication in mouse brains. Mice were inoculated
595 intracerebrally with 50 pfu each virus. Viral titers in the brain were determined at the indicated days
596 post-infection (d.p.i) using plaque assays. The virus detection limit for the assay was 10^2 pfu/ml.
597 Error bars represent standard deviations (n = 3). (D) Morbidity and mortality of the mice infected
598 with Sapporo-17-Io1 and Oshima 5-10. [a] Morbidity of mice was estimated by > 10% of weight loss. [b]

599 Number of sick mice/number of infected mice. [c] Number of dead mice/number of infected mice. *
600 Significant difference from Oshima 5-10 ($P < 0.05$).
601
602 **Fig. 7.** Histopathological features of the brain at 9 days post-infection. Each BALB/c mouse was
603 infected with 50 pfu of Sapporo-17-Io1 (A, C) or Oshima 5-10 (B, D). (A, B) The arrows indicate the
604 slight vascular cuffing. (C, D) TBEV antigens were detected using rabbit polyclonal antibodies
605 against the E protein. The arrows indicate the cells positive for viral antigens.
606
607 **Fig. 8.** (A) Construction of the chimeric viruses Oshima-IC/Sapporo-CME and Sapporo-
608 IC/Oshima-CME using infectious cDNA clones of TBEV Sapporo-17-Io1 and Oshima 5-10. (B)
609 Survival of mice infected with Sapporo-IC, Oshima IC and two chimeric viruses; Sapporo-
610 IC/Oshima-CME and Oshima-IC/Sapporo-CME. Mice were inoculated intracerebrally with 50 pfu (n
611 = 10) of each virus and monitored for 28 days. (C) Morbidity and mortality of the mice infected with
612 Sapporo-IC, Oshima-IC, Sapporo-IC/Oshima-CME, and Oshima-IC/Sapporo-CME. * Significant
613 difference from Oshima-IC ($P < 0.05$).
614

Figure. 1

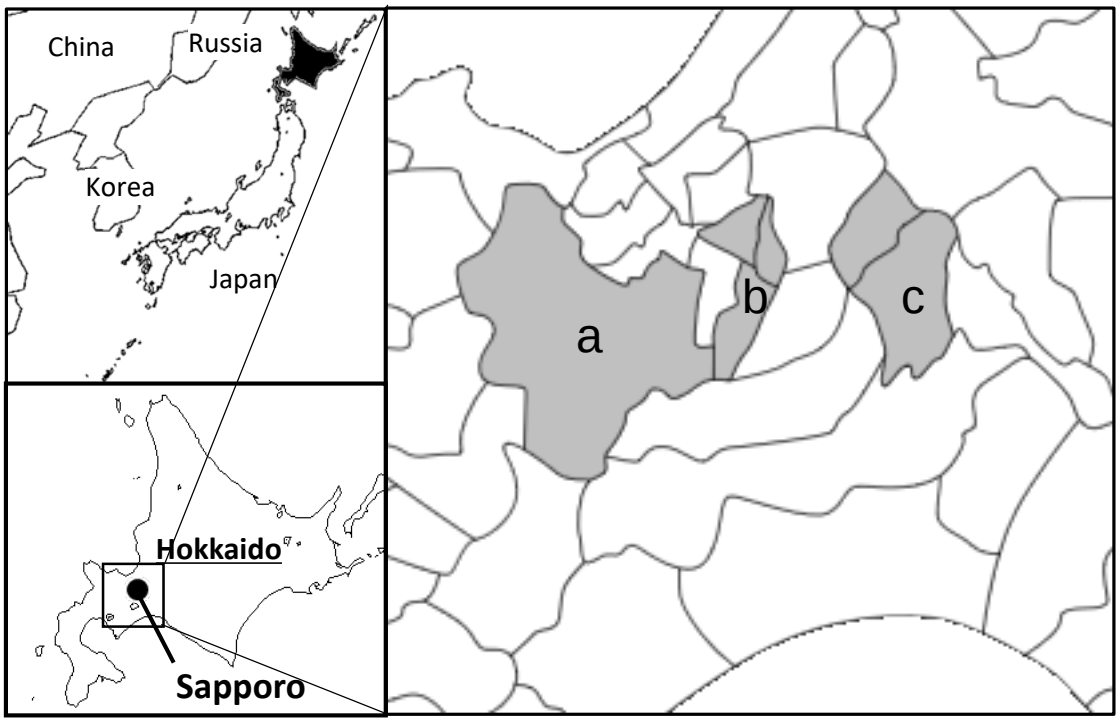


Figure. 2

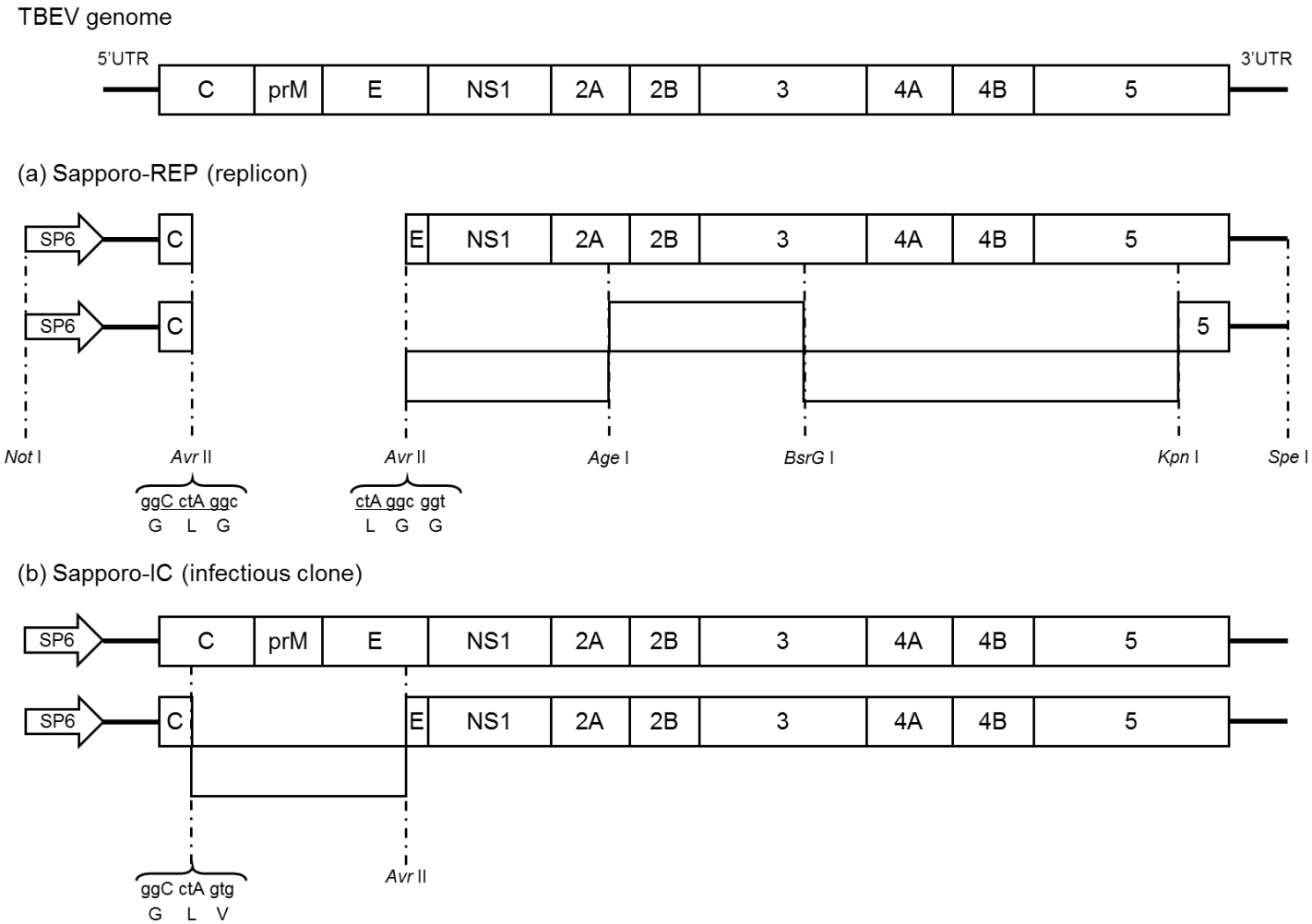

TBEV genome
5'UTR
C
prM
E
NS1
2A
2B
3
4A
4B
5
3'UTR
(a) Sapporo-REP (replicon)
SP6
C
SP6
C
E
NS1
2A
2B
3
4A
4B
5
5
Not I
Avr II
Avr II
Age I
BsrG I
Kpn I
Spe I
ggC ctA ggc
G L G
ctA ggc ggt
L G G
(b) Sapporo-IC (infectious clone)
SP6
C
prM
E
NS1
2A
2B
3
4A
4B
5
SP6
C
E
NS1
2A
2B
3
4A
4B
5
ggC ctA gtg
G L V
Avr II

Figure. 3

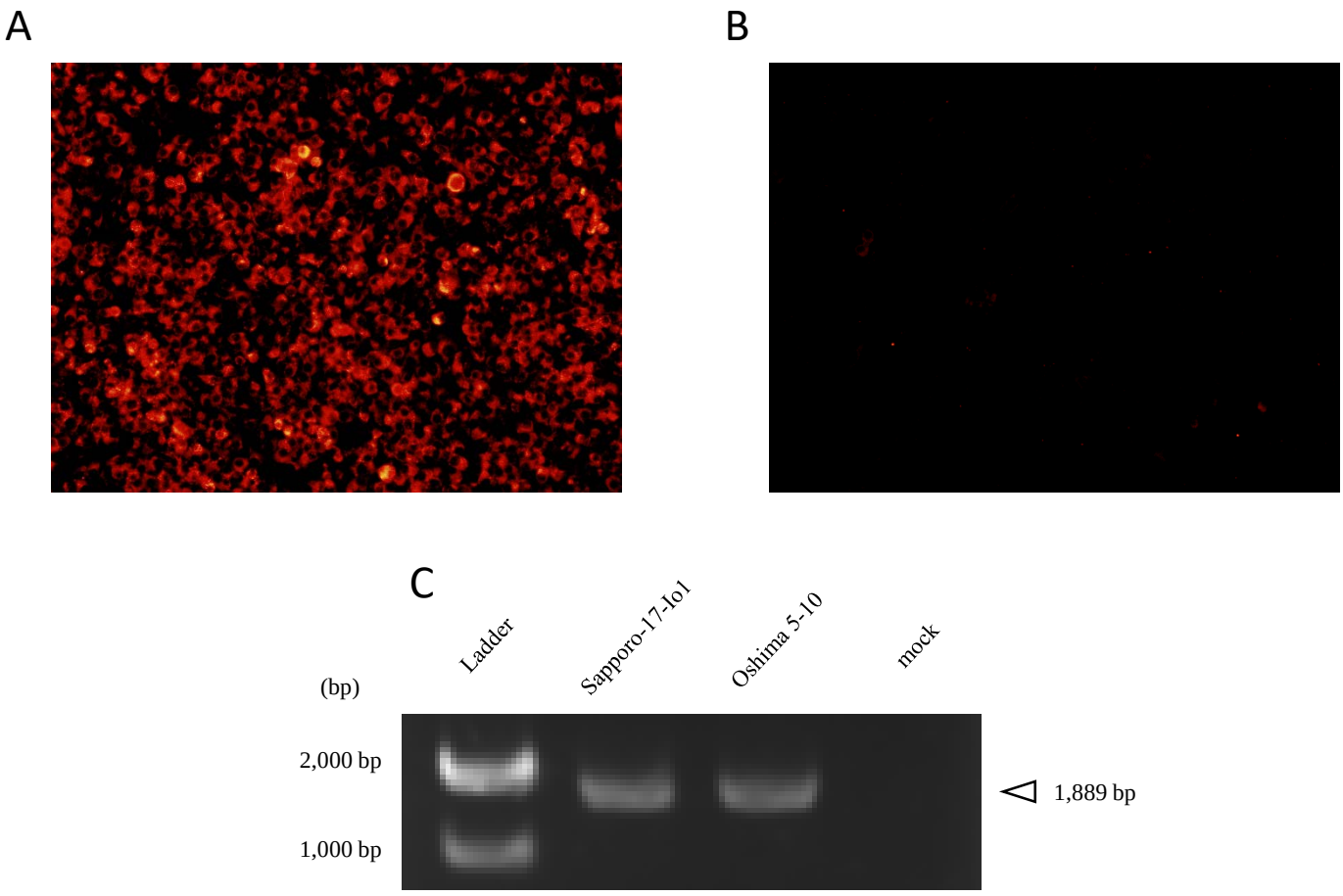

A
B
C
Ladder
Sapporo-17-Io1
Oshima 5-10
mock
(bp)
2,000 bp
1,000 bp
1,889 bp

Figure. 4

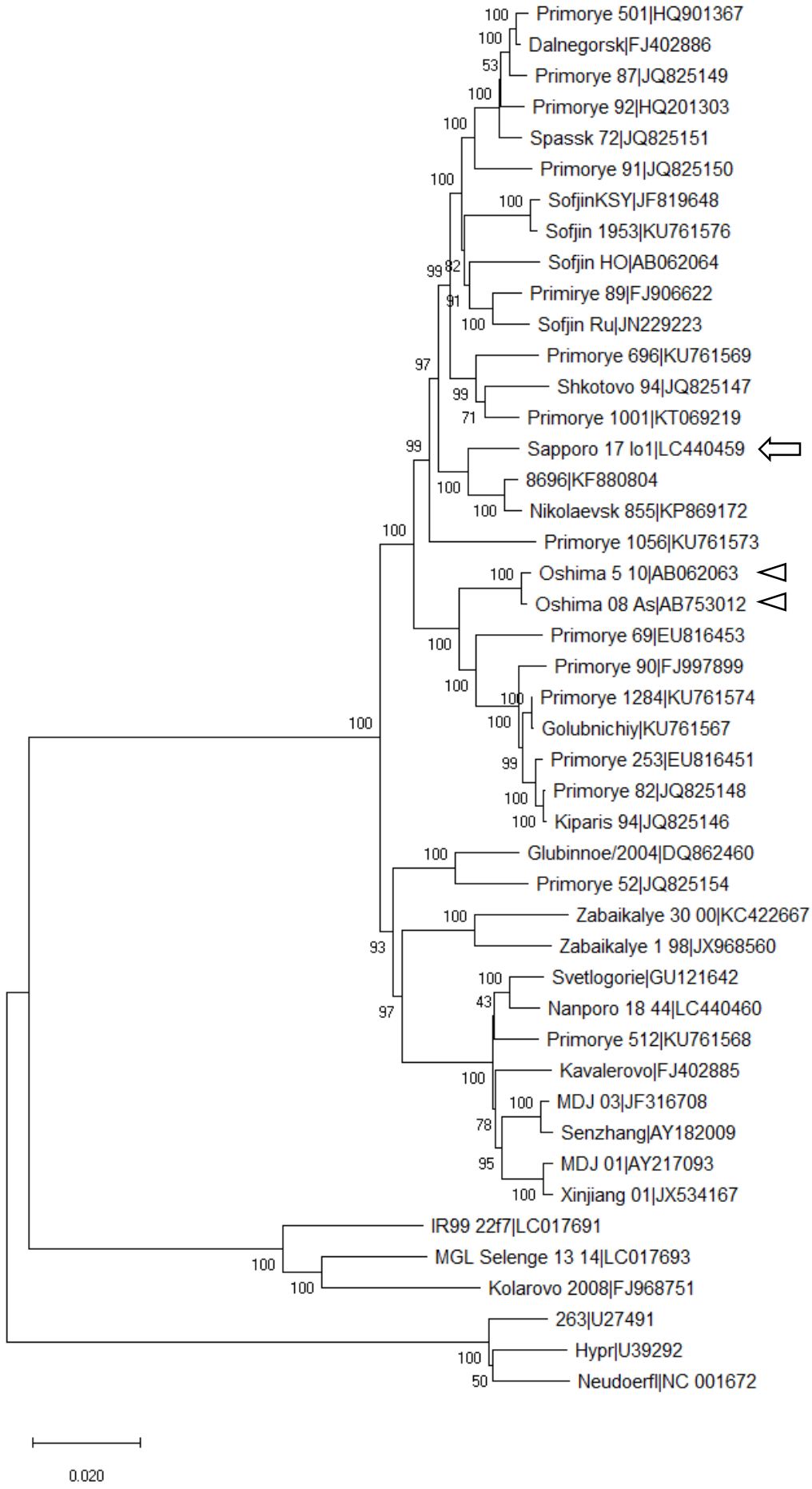

Primorye 501|HQ901367
Dalnegorsk|FJ402886
Primorye 87|JQ825149
Primorye 92|HQ201303
Spassk 72|JQ825151
Primorye 91|JQ825150
SofjinKSY|JF819648
Sofjin 1953|KU761576
Sofjin HO|AB062064
Primirye 89|FJ906622
Sofjin Ru|JN229223
Primorye 696|KU761569
Shkotovo 94|JQ825147
Primorye 1001|KT069219
Sapporo 17 lo1|LC440459
8696|KF880804
Nikolaevsk 855|KP869172
Primorye 1056|KU761573
Oshima 5 10|AB062063
Oshima 08 As|AB753012
Primorye 69|EU816453
Primorye 90|FJ997899
Primorye 1284|KU761574
Golubnichiy|KU761567
Primorye 253|EU816451
Primorye 82|JQ825148
Kiparis 94|JQ825146
Glubinnoe/2004|DQ862460
Primorye 52|JQ825154
Zabaikalye 30 00|KC422667
Zabaikalye 1 98|JX968560
Svetlogorie|GU121642
Nanporo 18 44|LC440460
Primorye 512|KU761568
Kavalerovo|FJ402885
MDJ 03|JF316708
Senzhang|AY182009
MDJ 01|AY217093
Xinjiang 01|JX534167
IR99 22f7|LC017691
MGL Selenge 13 14|LC017693
Kolarovo 2008|FJ968751
263|U27491
Hypr|U39292
Neudoerfl|NC 001672
0.020

Figure. 5

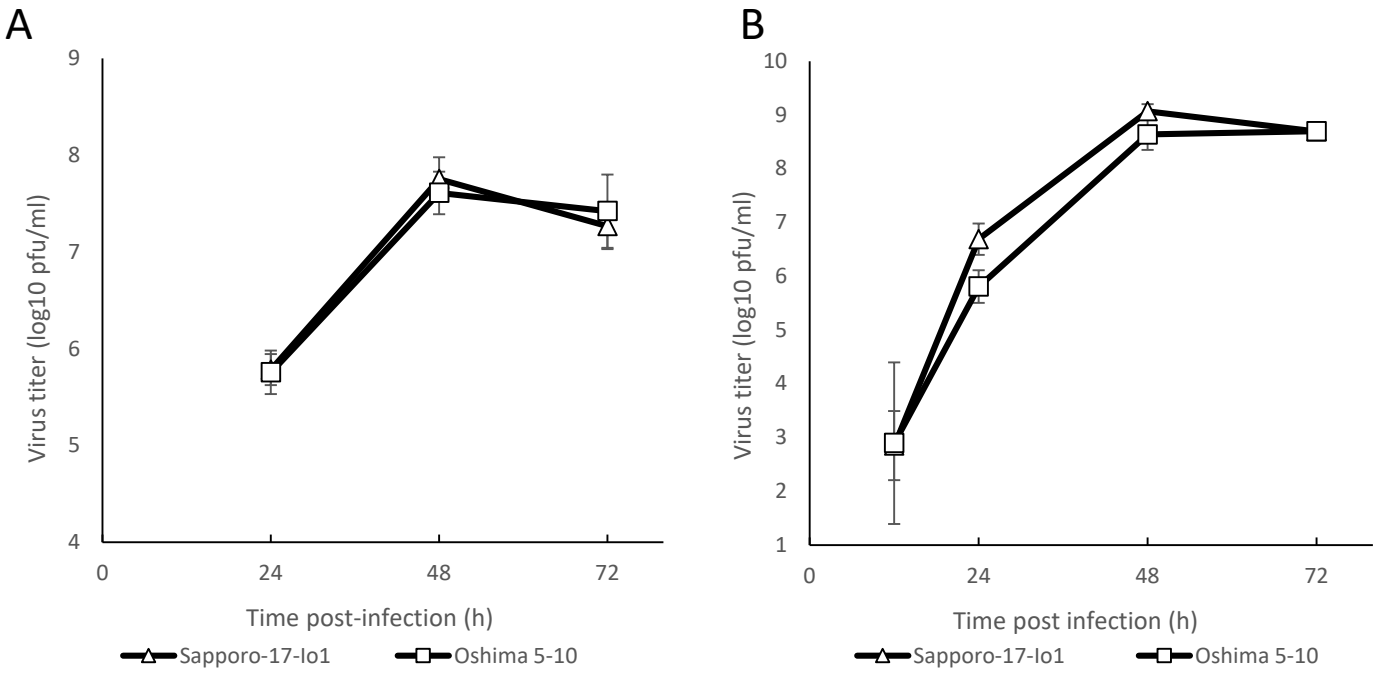
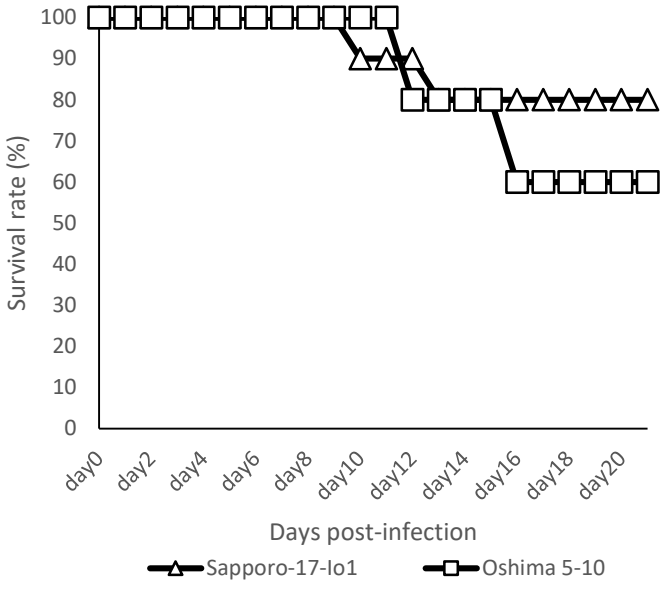

A
Virus titer (log10 pfu/ml)
9
8
7
6
5
4
0
24
48
72
Time post-infection (h)
Sapporo-17-Io1
Oshima 5-10
B
Virus titer (log10 pfu/ml)
10
9
8
7
6
5
4
3
2
1
0
24
48
72
Time post infection (h)
Sapporo-17-Io1
Oshima 5-10

Figure. 6

A



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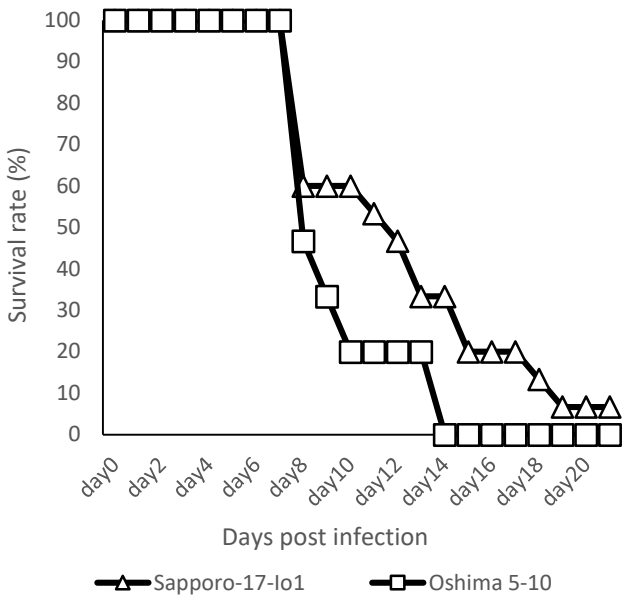

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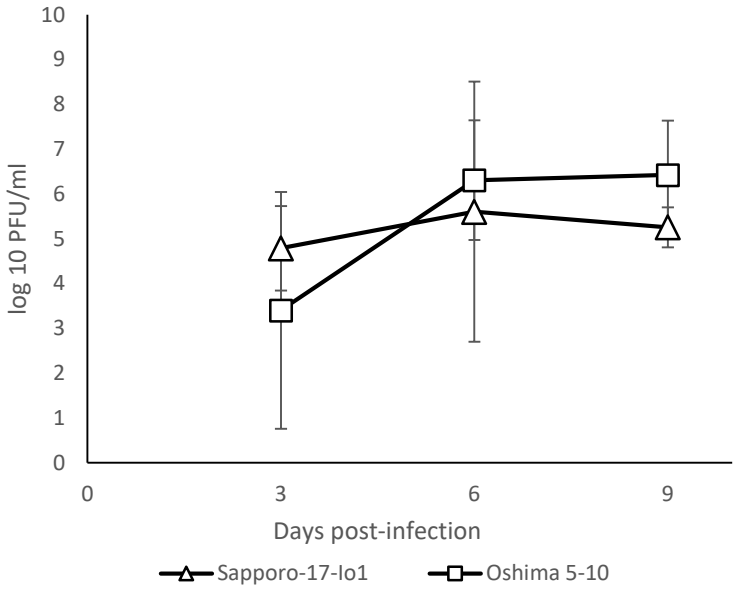


D

	Strain	Morbidity[a] (%)	Mortality (%)	Day of onset (days)	Survival time (days)
s.c.	Sapporo-17-Io1	20 (2/10)[b]	20 (2/10)[c]	9.5±0.7	11.5±2.1
	Oshima 5-10	80 (8/10)	40 (4/10)	12.1±2.1	14.0±2.3
i.c.	Sapporo-17-Io1	100 (15/15)	93 (14/15)	8.8±2.6	11.7±3.9*
	Oshima 5-10	100 (15/15)	100 (15/15)	7.1±1.1	9.6±2.4

Figure. 7

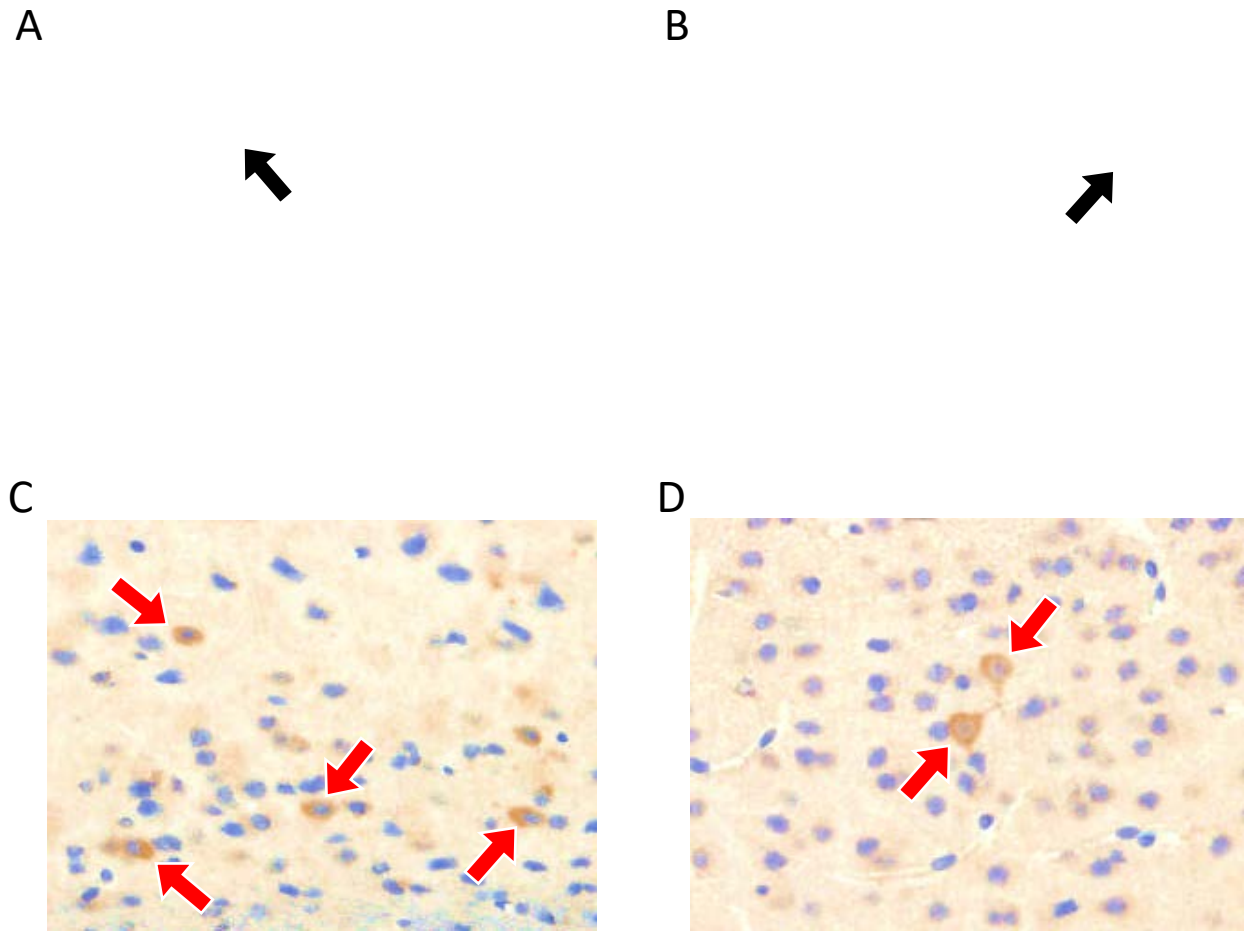
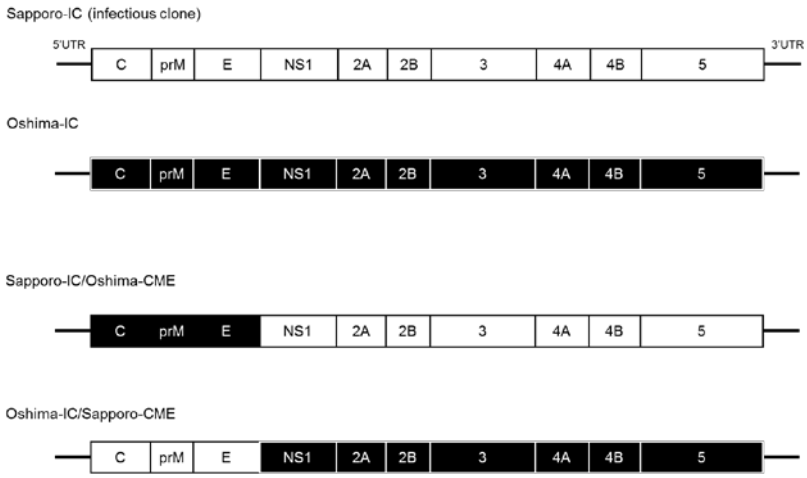
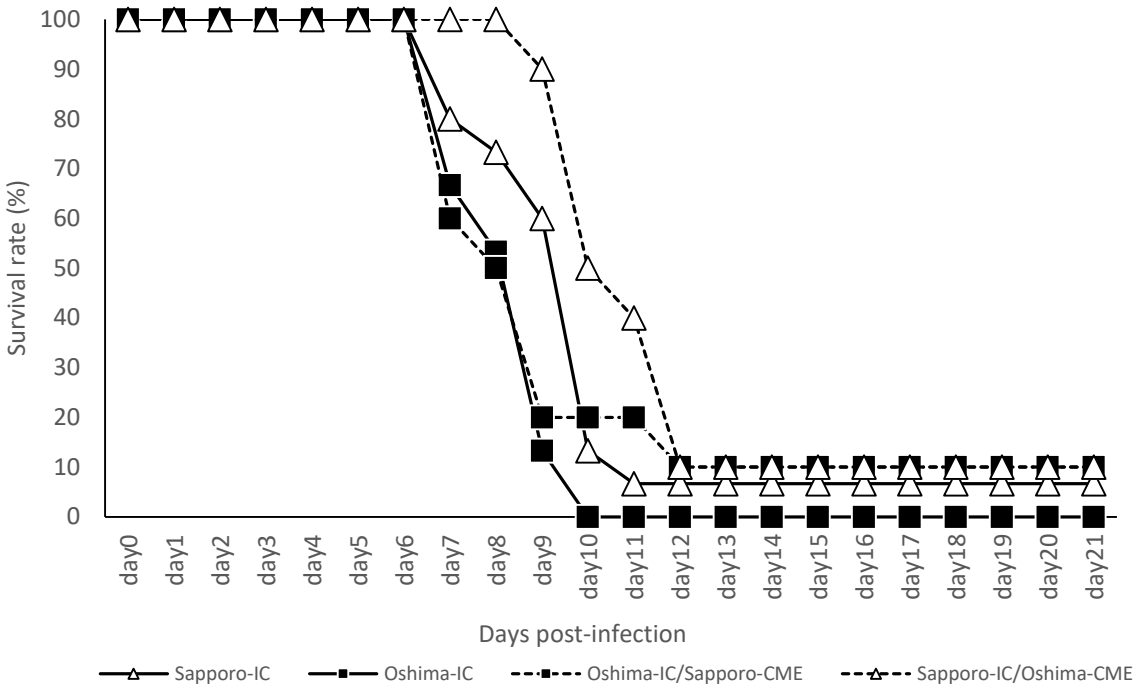


Figure. 8 A



B



C

	Strain	Morbidity (%)	Mortality (%)	Day of onset (days)	Survival time (days)
i.c.	Sapporo-IC	100 (15/15)	93.3 (14/15)	7.0±1.0	9.1±1.3*
	Oshima-IC	100 (15/15)	100 (15/15)	6.6±0.5	8.3±1.1
	Oshima-IC /Sapporo-CME	100 (10/10)	90 (9/10)	6.8±1.2	8.3±1.6
	Sapporo-IC /Oshima-CME	100 (10/10)	90 (9/10)	8.4±2.4	10.6±1.1*

Supplementary Table 1

Comparison of viral gene nucleotide sequences and amino acid sequences between Sapporo-17-Io1 and Oshima 5-10.

Gene	Homology (%) (differences/total)	
	Nucleotide	Amino Acid
5’ UTR	96.2 (5/131)	N/A
C	96.5 (10/288)	96.9 (3/96)
prM	95.1 (27/552)	97.8 (4/184)
E	96.1 (58/1488)	99.4 (3/496)
NS1	97.0 (32/1056)	98.9 (4/352)
NS2A	96.4 (25/690)	98.7 (3/230)
NS2B	97.2 (11/393)	100 (0/131)
NS3	95.9 (76/1863)	98.7 (8/621)
NS4A	95.5 (20/447)	100 (0/149)
NS4B	95.1 (37/756)	99.2 (2/252)
NS5	95.3 (127/2712)	98.8 (11/904)
3’ UTR	96.1 (28/725)	N/A
Total	95.9 (456/11101)	98.9 (38/3415)

Supplementary Table 2

Amino acid differences between Sapporo-17-Io1 and Oshima 5-10.

Nucleotide position	Amino acid position	Gene	Sapporo-17-Io1	Oshima 5-10
225	32	C	Q	R
321	64	C	K	N
324	65	C	L	S
426	99	prM	A	V
462	111	prM	L	V
930	267	prM	A	V
939	270	prM	A	V
1653	508	E	R	K
1887	586	E	M	V
2355	742	E	V	M
2631	834	NS1	V	L
2673	848	NS1	A	V
2736	869	NS1	L	F
2871	914	NS1	T	I
3669	1180	NS2A	K	R
4017	1296	NS2A	T	I
4188	1353	NS2A	S	V
4632	1501	NS3	D	E
4731	1534	NS3	S	F
4785	1552	NS3	I	V
4800	1557	NS3	A	V
4968	1613	NS3	R	G
5322	1731	NS3	N	S
5937	1936	NS3	M	T
6051	1974	NS3	S	G
7443	2438	NS4B	A	V
7587	2486	NS4B	G	S
7740	2537	NS5	V	A
7965	2612	NS5	K	R
8259	2710	NS5	L	F
8793	2888	NS5	R	K
9366	3079	NS5	I	T
9564	3145	NS5	S	T
9615	3162	NS5	Y	H
9738	3203	NS5	I	V
9996	3289	NS5	L	F
10158	3343	NS5	A	V
10248	3373	NS5	K	R