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Isolation and characterization of tick-borne encephalitis virus from *Ixodes persulcatus* in Mongolia in 2012.

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20 **Abbreviation**

21

22 BHK baby hamster kidney

23 CPE cytopathic effect

24 p.i. post infection

25 E envelope

26 IFA immunofluorescence assay

27 LGTV Langat virus

28 MOI multiplicity of infection

29 ORF open reading frame

30 PCR polymerase chain reaction

31 PFU plaque forming unit

32 RNA ribonucleic acid

33 RT-PCR reverse transcription polymerase chain reaction

34 TBE tick-borne encephalitis

35 TBEV tick-borne encephalitis virus

36 UTR untranslated region

37

38 **Abstract**

39

40 Tick-borne encephalitis virus (TBEV) is a zoonotic virus belonging to the genus *Flavivirus*, in the
41 family *Flaviviridae*. The virus, which is endemic in Europe and northern parts of Asia, causes severe
42 encephalitis. Tick-borne encephalitis (TBE) has been reported in Mongolia since the 1980s, but
43 details about the biological characteristics of the endemic virus are lacking. In this study, 680 ticks
44 (*Ixodes persulcatus*) were collected in Selenge aimag, northern Mongolia, in 2012. Nine Mongolian
45 TBEV strains were isolated from tick homogenates. A sequence analysis of the envelope protein
46 gene revealed that all isolates belonged to the Siberian subtype of TBEV. Two strains showed similar
47 growth properties in cultured cells, but their virulence in mice differed. Whole genome sequencing
48 revealed only thirteen amino acid differences between these Mongolian TBEV strains. Our results
49 suggest that these naturally occurring amino acid mutations affected the pathogenicity of Mongolian
50 TBEV. Our results may be an important platform for monitoring TBEV to evaluate the
51 epidemiological risk in TBE endemic areas of Mongolia.

52

53 **Keywords:** Flavivirus, Tick-borne encephalitis, Mongolia

54

Introduction

Tick-borne encephalitis virus (TBEV), a member of the genus *Flavivirus* within the *Flaviviridae* family, causes severe encephalitis in humans. Tick-borne encephalitis (TBE) is a zoonotic disease and is endemic in Europe, Russia, and northern parts of Asia, including Japan (Lindquist and Vapalahti, 2008; Suss, 2008; Takashima et al., 1997). TBEV is maintained between ticks (family *Ixodes*) and wild vertebrate hosts in nature. Humans are not involved in the natural transmission of TBEV and are only accidental hosts. Although, vaccines are currently available, TBE has a significant impact on public health in these endemic regions (Kunz and Heinz, 2003).

TBEV has been divided into three subtypes: the European subtype, the Siberian subtype and the Far-Eastern subtype (Ecker et al., 1999). These subtypes cause different symptoms and mortality (Gritsun et al., 2003). The European subtype, which is distributed throughout Europe, causes a biphasic fever and milder form of encephalitis, and the mortality rate is up to 2% (Dumpis et al., 1999; Suss, 2008). The distribution range of the Far-Eastern subtype covers Eastern Russia, northern China, and Japan. Infection with this subtype of TBEV provokes the most severe neural disorder, including encephalitis and meningoencephalitis, and the mortality rate is up to 30% (Ecker et al., 1999). The Siberian subtype is widely distributed throughout Russia and the case mortality rate is 6–8%. Despite the milder form of encephalitis caused by Siberian subtype compared to the Far-Eastern subtype, humans infected with the Siberian subtype often develop chronic disease (Gritsun et al., 2003).

The ranges of the Far-Eastern and the Siberian subtypes is expected to overlap in Mongolia between northern China and the Asian part of Russia, respectively (Kulakova et al., 2012; Zhang et al., 2012). Mongolia is also a TBE endemic region (Walder et al., 2006). Severe TBE cases have been reported since the 1980s in Selenge aimag and Bulgan aimag (near the border with Russia) (Frey et al., 2012). In Bulgan aimag, the viral genome was detected in a patient in 2008 and from ticks in 2010 and these viral genes were clustered within the Far-Eastern subtype and the Siberian

subtype, respectively (Frey et al., 2012; Khasnatinov et al., 2010). However, minimal data are available concerning the biological characteristics of Mongolian TBEV strains (e.g., virulence and viral multiplication).

In this study, we collected ticks (*Ixodes persulcatus*) from Selenge aimag in Mongolia, and isolated the TBEV. We detected the TBEV antigens and genomic ribonucleic acid (RNA) in cell cultures inoculated with tick homogenates. Sequencing revealed that the isolated viruses belonged to the Siberian subtype of TBEV. Viral growth and plaque morphology were assessed and the pathogenicity of the viral isolates was analyzed in a mouse model.

Materials and Methods

Tick collection and virus isolation

TBEV strains were isolated from ticks (*I. persulcatus*) collected in Bugant village, Selenge aimag, in northern Mongolia, in 2012 (Fig. 1). In total, 680 ticks were collected by dragging flannel sheets over the vegetation and pooled into groups of 20-30 ticks. The pools were washed with ethanol and homogenized in phosphate buffered saline with a pestle. Each homogenized suspension was centrifuged, and the supernatant was collected and stored at -80°C until the inoculation step.

Baby hamster kidney (BHK) cells were grown in 24-well plates. Then inoculated with the supernatants collected in the previous step, and incubated at 37°C under 5% CO_2 for 1 h. After 2–4 days, the cells were checked for cytopathic effect (CPE) and supernatants from cells showing a CPE were harvested and stored at -80°C . The viruses in these samples were identified by immunofluorescence assay (IFA) using anti-tick borne flavivirus antibodies and reverse transcription polymerase chain reaction (RT-PCR). All stock viruses were propagated once in BHK cells.

Detection of viral antigens

The tick homogenates were inoculated onto a monolayer of BHK cells. After 3 days of

incubation at 37°C under 5% CO₂, the 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 hyper-immune murine ascites fluid from Langat virus (LGTV) infected mice (which is cross-reactive to TBEV), followed by Alexa 555-conjugated anti-mouse immunoglobulin G antibodies (Invitrogen, Carlsbad, CA).

RT-PCR

Viral RNA was extracted from BHK cells using ISOGEN (Nippon Gene, Tokyo, Japan) and reverse-transcribed with random primers using M-MLV Reverse Transcriptase (Life Technologies, Carlsbad, CA). The TBEV-specific sequence was amplified with Platinum *Taq* polymerase (Invitrogen). To amplify the envelope (E) protein gene of TBEV, universal primers for the Far-Eastern and the Siberian subtypes of TBEV were designed and used. (Mongolia-F: 5'-GGTYATGGARGTYRCRTTCTCTCG-3', and Mongolia-R: 5'-TCCCAGGCGTGYTCTCCKATCACTGT-3').

TBE viral gene sequencing

The nucleic acid sequences of the viral genomes were determined by direct sequencing. The cycle sequencing reactions were performed using a BigDye™ Terminator Cycle Sequencing Kit (Life Technologies), and the sequences were determined with a 3130 Genetic Analyzer (Life Technologies). The primers used for sequencing were shown in Supplementary Table 1.

Phylogenetic analysis

A phylogenetic analysis was performed using the complete E gene and the complete full sequence genomes of the TBEV strains. LGTV was used as the outgroup. Genetyx version 8 was used to generate the multiple alignments. MEGA 6 (<http://www.megasoftware.net/>) was used to generate phylogenetic trees by the neighbor-joining method. The reliability of the dendrogram was

133 evaluated using 500 bootstrap replicates. The GenBank Accession Numbers of the sequences were
134 shown in Fig. 3.

135

136 **Growth curve and plaque morphology assays in cell culture**

137 For titration, BHK cell monolayers prepared in 12-well plates were incubated with serial
138 dilutions of virus for 1 h, and then overlaid with minimal essential medium containing 2% fetal
139 bovine serum and 1.5% carboxymethyl cellulose and incubated for 4 days. The cells were then fixed
140 and stained with crystal violet (0.25% in 10% buffered formalin) to visualize plaques. Plaques were
141 counted and expressed as plaque-forming units (PFU).

142 Subconfluent BHK cells were grown in 12-well plates then inoculated with virus at a
143 multiplicity of infection (MOI) of 0.01 PFU/ml. The cells were incubated at 37°C under 5% CO₂.
144 Supernatants were harvested at 12, 24, 48, and 72 h post-infection (p.i.) and stored at -80°C until
145 using for titration.

146

147 **Animal model**

148 Each virus was inoculated subcutaneously at 10³ PFU into ten 5-week-old female C57BL/6J
149 mice (Japan SLC, Shizuoka, Japan). Surviving mice were monitored for 28 days p.i. to determine
150 survival curves and mortality rates. Onsets of disease were estimated at 10% weight loss compared
151 with the weight before virus infection. For the analysis of viral distribution in tissues, three to four
152 mice were sacrificed on 3, 6, 9, 11 days p.i., and sera and brains were collected. Organs were
153 individually weighted, homogenized, and prepared as 10% (w/v) suspensions in phosphate buffered
154 saline with 10% fetal bovine serum. Suspensions were then clarified by centrifugation (5,000 rpm for
155 5min at 4°C), and the supernatants were titrated.

156 All animal experiments were performed in accordance with the Fundamental Guidelines for
157 Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions
158 under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

159 Experimental protocols were approved by the Animal Care and Use Committee of Hokkaido
160 University.

161

162 **Results**

163

164 **Isolation and identification of TBEV in Mongolia**

165 Nine strains of TBEV were isolated from *I. persulcatus* collected in Bugant village, Selenge
166 aimag, Mongolia. BHK cells were inoculated with tick homogenates and the supernatants were blind
167 passaged again. After incubation period for 2–4 days, CPE were observed in cells inoculated with
168 nine homogenate pools. Viral-specific antigens and bands were detected in the cells by IFA and
169 RT-PCR, respectively (Fig. 2). These isolates were identified as TBEV and designated as
170 MGL-Selenge-13 strains (-5, -12, -13, -14, -15, -18, -19, -21, and -25).

171

172 **Genetic analysis of the isolated TBEV strains**

173 The nucleotide sequences of the viral E protein gene from the seven isolated
174 MGL-Selenge-13 strains (-12, -13, -14, -15, -18, -19, and -21) and the complete genomic sequences
175 of MGL-Selenge-13-12 and MGL-Selenge-13-14 were determined. A phylogenetic tree of the viral E
176 gene and the open reading frame (ORF) is shown in Fig. 3. All isolated strains were classified as the
177 Siberian subtype of TBEV and formed a similar cluster. The GenBank accession numbers of the
178 viruses used in this study were shown in Fig. 3.

179 The nucleotide and amino acid sequence of the viral E gene were compared with
180 MGL-Selenge-13-12, MGL-Selenge-13-14, MucAr M14/10 (isolated in Mongolia), M92 (isolated in
181 Mongolia), and IR99 2f7 (isolated in Russia) (Table 1). All strains highly homologous (>90%) in
182 both their nucleotide and amino acid sequences. The full length sequences of MGL-Selenge-13-12
183 and MGL-Selenge-13-14 (11106 nt) were also compared. The nucleotide homology was 99.1%
184 (11005 nt /11106 nt) in the complete sequences, including the 5'- and 3'- untranslated regions

185 (UTRs). Nucleotide substitutions were observed in the 5'-UTR (one nucleotide) and 3'-UTR (seven
186 nucleotides), but no deletions or insertions were observed in these regions. The amino acid
187 differences were located only in the viral E (residues 580, 597, and 631), NS3 (residues 1743, 1992,
188 and 2046), and NS5 (residues 2623, 3221, 3223, 3352, 3357, 3403, and 3409) genes (Table 2).

189

190 **Growth properties and pathogenicity of MGL-Selenge-13-12 and MGL-Selenge-13-14**

191 The growth properties of MGL-Selenge-13-12 and MGL-Selenge-13-14 were compared
192 with those of IR99 2f7 by monitoring viral release after infection. BHK cells were infected with each
193 virus at an MOI of 0.01. Viruses were harvested at 12, 24, 48, and 72 h p.i., and the yields quantified
194 using a plaque assay. The virus growth titers were similar among the MGL strains (Fig. 4-A).
195 MGL-Selenge-13-12 and MGL-Selenge-13-14 strains showed similar plaque size, while plaque size
196 of IR99 2f7 was relatively large (Fig. 4-B).

197 The pathogenicity of MGL-Selenge-13-12 and MGL-Selenge-13-14 was examined and
198 compared with that of the other Siberian subtype strain, IR99 2f7, in a mouse model. Mice were
199 infected subcutaneously with 10^3 PFU/mouse of each virus strain and survival rates were recorded
200 over 28 days (Fig. 5). All mice infected with IR99 2f7 and MGL-Selenge-13-12 showed clinical
201 symptoms such as a hunched posture, weight loss, ruffled fur, and general malaise. The mice with
202 more severe disease showed neurological symptoms, including paralysis and loss of balance. The
203 mice infected with MGL-Selenge-13-14 showed significantly reduced symptoms compared to the
204 mice infected with IR99 2f7 and MGL-Selenge-13-12 ($P<0.05$). The morbidity and mortality rates
205 were significantly lower for mice infected with MGL-Selenge-13-14 than those for mice infected
206 with IR99 2f7 or MGL-Selenge-13-12 (Fig. 5). The survival time and time to the onset of disease
207 were longer for the MGL-Selenge-13-14 infected mice than for the IR99 2f7 or MGL-Selenge-13-12
208 infected mice (Table 3). These data indicate that MGL-Selenge-13-14 is less virulent than
209 MGL-Selenge-13-12 in mice.

210 To examine the viral replication in organs, MGL-Selenge-13-12 and MGL-Selenge-13-14

strains were inoculated into mice. Viral multiplication in MGL-Selenge-13-12-infected mice sera were observed from 3 days p.i., but not in MGL-Selenge-13-14-infected mice sera (Fig. 6). The virus was detected in the brain from 9 days p.i. in all MGL-Selenge-13-12-infected mice and only one mice infected with MGL-Selenge-13-14 at 9 and 11 days p.i. respectively. The virus titer reached 8.9×10^7 PFU/ml at 11 days p.i. in the mice inoculated with MGL-Selenge-13-12 and was significantly higher than that in the mice infected with the MGL-Selenge-13-14 (Fig. 6).

Discussion

In this study, nine TBEVs (MGL-Selenge-13) were isolated from *I. persulcatus* collected in Bugant village, Selenge aimag, Mongolia (Figs. 1, and 2). The TBEV detection rate in ticks (1.3%) was similar to that in a previous study (1.6%) in Mongolia (Frey et al., 2012). TBEV infected human cases were found mainly in northern Mongolia, especially Selenge aimag (Walder et al., 2006). The Siberian subtype (92M and MucAr M14/10) of TBEV was also detected from a patient and ticks in northern Mongolia (Frey et al., 2012; Khasnatinov et al., 2010). These results showed that the Siberian subtype of TBEV is endemic in northern Mongolia. This area is located next to the Asian part of Russia, which is known to be a severe TBEV endemic region (Hayasaka et al., 2001). Human activity may have contributed to the transmission of TBEV from Siberian Russia to Mongolia via the Trans-Siberian railway (Frey et al., 2012; Kovalev et al., 2009). Additionally, Mongolia is an important place for wild bird migration. There are transmission directions of influenza A virus between Russia and Mongolia via birds migration (Kang et al., 2011). Surveillance in Russia showed wild birds were bitten by ticks infected with TBEV (Mikryukova et al., 2014). It is possible that wild birds may contribute to the transmission of TBEV into Mongolia.

From our phylogenetic analysis (Fig. 3-A), the MGL-Selenge-13 strains were classified in the same subcluster as the Siberian subtype. Within the cluster of the Siberian subtype of Mongolian

237 TBEV, the strains diverged into two subclusters. The MGL-Selenge-13 strains were classified in the
238 same subcluster as the 92M strain but not the MucAr M14/10 strain. The MucAr M14/10 strain was
239 in a subcluster with the IR99 2f7 strain isolated from Irkutsk (Hayasaka et al., 2001). It has been
240 reported that several subclusters of Siberian TBEV are endemic in Russia (Hayasaka et al., 2009).
241 Our results suggest that at least two subclusters of TBEV invaded from Russia into Mongolia,
242 independently.

243 The biological characteristics of the MGL-Selenge-13-12 and the MGL-Selenge-13-14
244 strains were compared (Fig. 4). The virus titer in BHK cells and the plaque size were almost similar
245 between the MGL-Selenge-13-12 and the MGL-Selenge-13-14 strains. However, the virulence of the
246 MGL-Selenge-13-14 strain in mice was significantly lower than that of the MGL-Selenge-13-12
247 strain (Fig. 5). Our results indicated the plaque morphology and size of the Mongolian isolates were
248 not directly correlated with the neuroinvasiveness as shown in a previous study of TBEV isolates
249 from Switzerland by Gaeumann (Gaeumann et al., 2011). The death of mice infected with the
250 MGL-Selenge-13-14 was delayed compared with that of mice infected with MGL-Selenge-13-12,
251 and several mice recovered after the onset of disease. It was previously reported that a combination
252 of central nervous system pathology and systemic inflammatory responses were involved in the late
253 death of mice infected with some strains of TBEV (Hayasaka et al., 2009). It is possible that these
254 types of pathological features contributed to the difference in virulence between the
255 MGL-Selenge-13-12 and the MGL-Selenge-13-14 strains.

256 Increased viral multiplication was observed in the blood and brain of the mice infected with
257 MGL-Selenge-13-12 and it was significantly higher than those of MGL-Selenge-13-14-infected mice,
258 although they showed similar growth properties in BHK cells (Figs. 4-A, 6). These results indicated
259 that the induction of the host immune responses might be different in the infection of the Mongolian
260 isolates and that it affected the viral multiplication in the organs leading to the different virulence in
261 the mice.

262 The complete genomic sequence of the MGL-Selenge-13-12 and the MGL-Selenge-13-14

263 strains differed by thirteen amino acids. Previous studies showed that naturally occurring mutations
264 affect the pathogenicity of TBEV in nature (Formanova et al., 2015; Kentaro et al., 2013). In the viral
265 E gene, it roles virus entry, three amino acids differences were located in domains II and III. In NS3,
266 three amino acids differences were located in the C-terminal domain (residues 180-618), which
267 possesses helicase activity (Lescar et al., 2008). One amino acid difference was located in the
268 N-terminal Methyltransferase (MTase) domain and six amino acids differences were in the
269 C-terminal RNA-dependent RNA polymerase (RdRp) domain in NS5 (Egloff et al., 2002; Selisko et
270 al., 2006). Interestingly, the Cysteine residue at position 3,221 is strictly conserved within family
271 *Flavivirdae* (Selisko et al., 2006). It might be possible that the cysteine-to-tryptophan substitution in
272 MGL-Selenge-13-14 strain affected the viral growth in mice (Table 2). Previous studies have shown
273 that NS5 of flavivirus has an interferon antagonist activity which suppress innate immune responses
274 (Best et al., 2005; Lin et al., 2006). The amino acids differences between MGL strains in NS5 could
275 be involved in the alternation of the interferon antagonism. Several studies reported that the mutation
276 of each of these proteins affected the virulence of TBEV (Belikov et al., 2014; Goto et al., 2003;
277 Mandl et al., 2000; Rumyantsev et al., 2006; Yoshii et al., 2014). However, the different amino acids
278 observed in the two Mongolian isolates have not been reported to be involved in the virulence in
279 mice. Identification of the viral factor (mutation) responsible for the difference in virulence between
280 the MGL-Selenge-13-12 and the MGL-Selenge-13-14 strains will lead to further our understanding
281 of the functions of the viral protein in the pathogenicity of the Siberian subtype of TBEV.

282 In summary, we newly isolated the Siberian subtype of TBEV in Selenge aimag in Mongolia.
283 Several strains showed different levels of virulence in a mouse model, indicating that a few naturally
284 occurring mutations affect the virulence of the endemic strains in Mongolia. Minimal data are
285 available about TBEV, which is endemic in Mongolia due to the lack of established diagnostic
286 systems for TBE. Moreover, the Far-Eastern subtype of TBEV was also detected in other survey in
287 Mongolia (Khasnatinov et al., 2010). To determine the distribution of TBEV in Mongolia, additional

288 epidemiological studies are necessary. Our results could be an important platform for monitoring
289 TBEV to evaluate the epidemiological risk in TBE endemic areas of Mongolia.

290

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384

385 **Figure legends**

386

387 Fig. 1. Geographical distribution of the study area.

388 Fig. 2. Detection of TBEV specific antigens and RNA. Immunofluorescence analysis of BHK cells.

389 BHK cells were inoculated with the supernatant from TBEV positive tick homogenates
390 (MGL-Selenge-13-12) (A) or a mock sample (B) and TBEV antigen was detected with anti-tick
391 borne flavivirus antibodies. (C) The E protein gene of TBEV (1488 bp) were amplified by RT-PCR
392 from cells inoculated with TBEV positive tick homogenates (MGL-Selenge-13- 5, -12, -13, -14, -15,
393 -18, -19, -21, and 25) and mock treated cells (N).

394 Fig. 3. (A) Phylogenetic tree of TBEV strains based on 1488 nucleotides of the viral E gene. LGTV
395 was used as an outgroup. The percentage of bootstrap values are shown next to the branches.

396 *Isolated TBEV strains in this study. Accession numbers are shown after the virus strains. (B)
397 Phylogenetic tree of TBEV strains based on the ORF of the viral gene. LGTV was used as an
398 outgroup. Bold letters indicate the strains isolated in Mongolia.

399 Fig. 4. (A) Comparison of the growth curves of IR99 2f7, MGL-Selenge-13-12, and
400 MGL-Selenge-13-14.

401 A monolayer of BHK cells was infected with each virus at a multiplicity of infection (MOI) of 0.01.
402 At each time point, the medium was harvested and virus titers were determined using a plaque assay
403 in BHK cells. (B) Plaque morphology of IR99 2f7, MGL-Selenge-13-12, and MGL-Selenge-13-14 in
404 BHK cells. BHK cells were stained with crystal violet (0.1%) at 96h p.i.

405 Fig. 5. Survival of mice inoculated with TBE viral strains IR99-2f7, MGL-Selenge-13-12, and
406 MGL-Selenge-13-14. Mice were inoculated subcutaneously with 10^3 PFU of each virus and
407 monitored for 28 days. * MGL-Selenge-13-14 showed significant differences in survival rate
408 compared with IR99 2f7 and MGL-Selenge-13-12 ($P<0.05$). Survival rates were calculated using
409 the Kaplan-Meier method and P -values for the differences in survival rates were calculated using

410 log-rank tests.

411 Fig. 6 Viral multiplication in mice organs. Mice were inoculated subcutaneously with 10^3 PFU of
412 each virus. Virus titers in the blood and the brain at the indicated days after infection were
413 determined by plaque assays. The limits of virus detection for the assay was 10^2 PFU/ml. Error bars
414 represent the standard deviation (n=3 or 4). Asterisks indicate significant differences compared with
415 MGL-Selenge-13-12 and MGL-Selenge-13-14 * (P<0.05) ** (P<0.01).

Table 1. Comparison of viral E gene nucleotide sequences and amino acid sequences

Nucleotide	Amino acid											
			MGL-Selenge-13-12		MGL-Selenge-13-14		McuAr M14/10		92M		IR99 2f7	
	MGL-Selenge-13-12		99.4% (493/496 aa)		99.2% (492/496 aa)		98.8% (490/496 aa)		99.0% (491/496 aa)			
	MGL-Selenge-13-14		98.4% (1464/1488 bp)		99.4% (493/496 aa)		99.0% (491/496 aa)		99.2% (492/496 aa)			
	MucAr M14/10		94.6% (1407/1488 bp)		93.9% (1397/1488 bp)		99.6% (494/496 aa)		99.8% (495/496 aa)			
	92M		96.2% (1431/1488 bp)		96.2% (1432/1488 bp)		94.6% (1407/1488 bp)		99.4% (493/496 aa)			
	IR99 2f7		94.2% (1401/1488 bp)		93.8% (1395/1488 bp)		97.0% (1439/1488 bp)		94.6% (1407/1488 bp)			

Table 2. Amino acid differences between MGL-Selenge-13-12 and MGL-Selenge-13-14

Amino acid Position	Gene	MGL-Selenge-13-12	MGL-Selenge-13-14
580	Envelope	Arg	Lys
597	Envelope	Asn	Thr
631	Envelope	Asp	Glu
1743	NS3	Ser	Gly
1992	NS3	Val	Leu
2046	NS3	Pro	Ser
2623	NS5	Ala	Thr
3221	NS5	Cys	Trp
3223	NS5	His	Arg
3352	NS5	Leu	Pro
3357	NS5	Ile	Met
3403	NS5	Leu	Ser
3409	NS5	Leu	Met

Table 3. Mortality and morbidity of the virus infected mice

strain	morbidity ^a (%)	mortality (%)	day of onset (days)	survival time (days)
IR99 2f7	100 (10/10) ^b	100 (10/10) ^c	9.4 ± 1.8	12.7 ± 2.9
MGL-Selenge-13-12	100 (10/10)	80 (8/10)	10.3 ± 1.5	13.8 ± 1.6
MGL-Selenge-13-14	50 (5/10)	40 (4/10)	14.0 ± 4.3	17.3 ± 4.6

^a Morbidity of mice was estimated by >10% of weight loss.

^b Number of sick mice / number of infected mice.

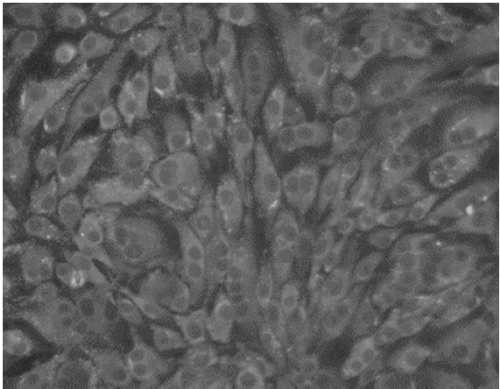
^c Number of dead mice / number of infected mice.

Fig. 1.

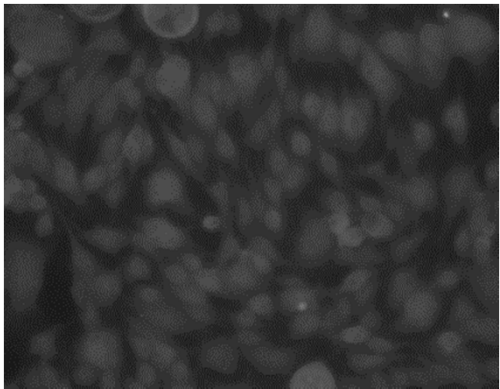


Fig. 2.

A



B



C

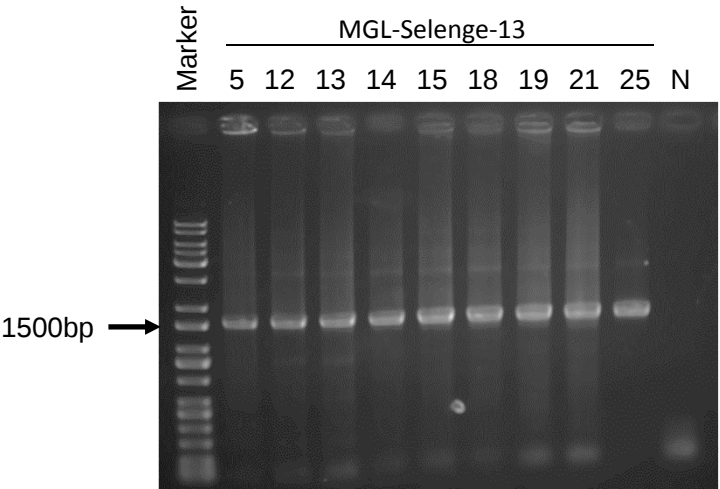


Fig. 3-A

E protein gene (1,488 nt)

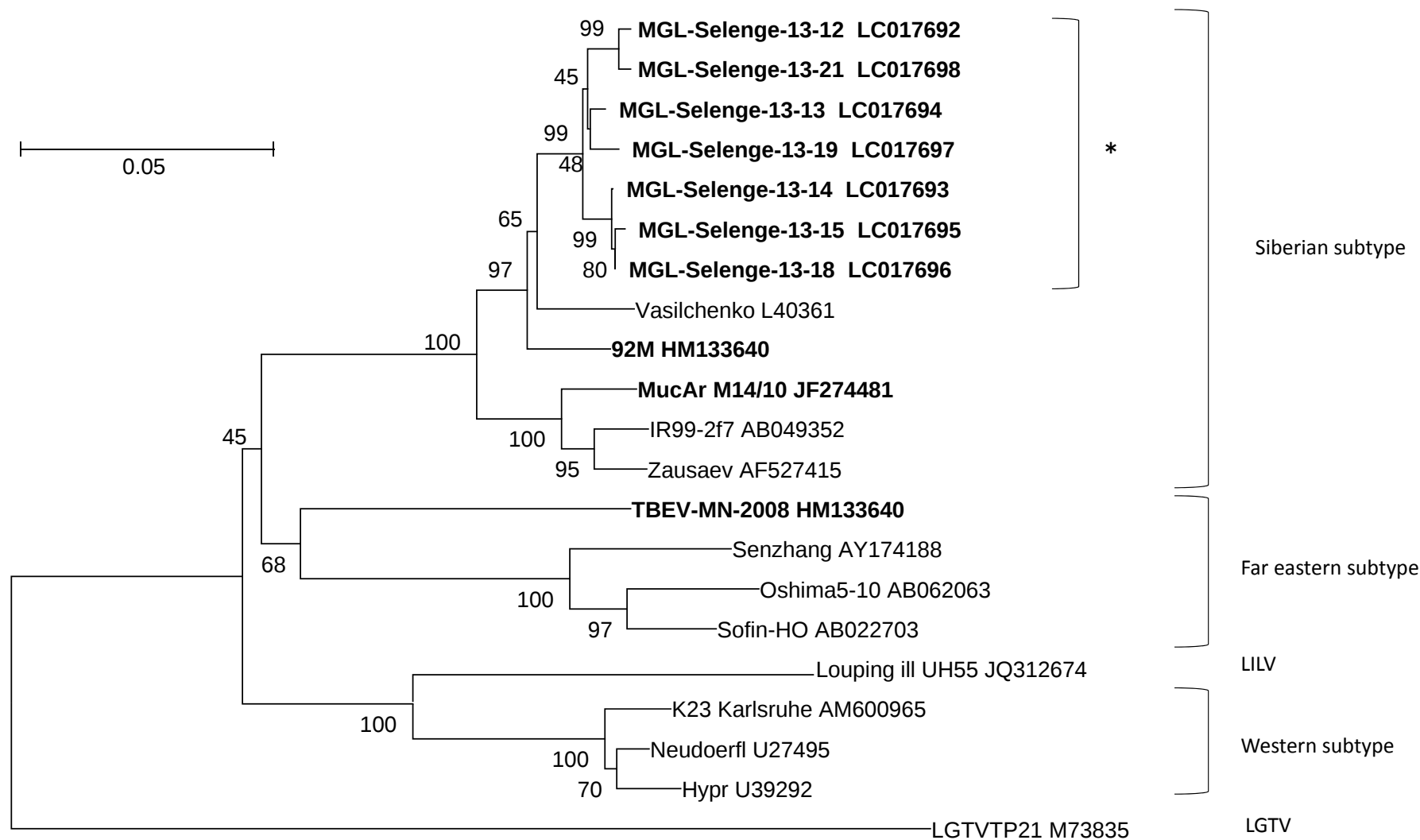


Fig. 3-B

Complete ORF (11,106 nt)

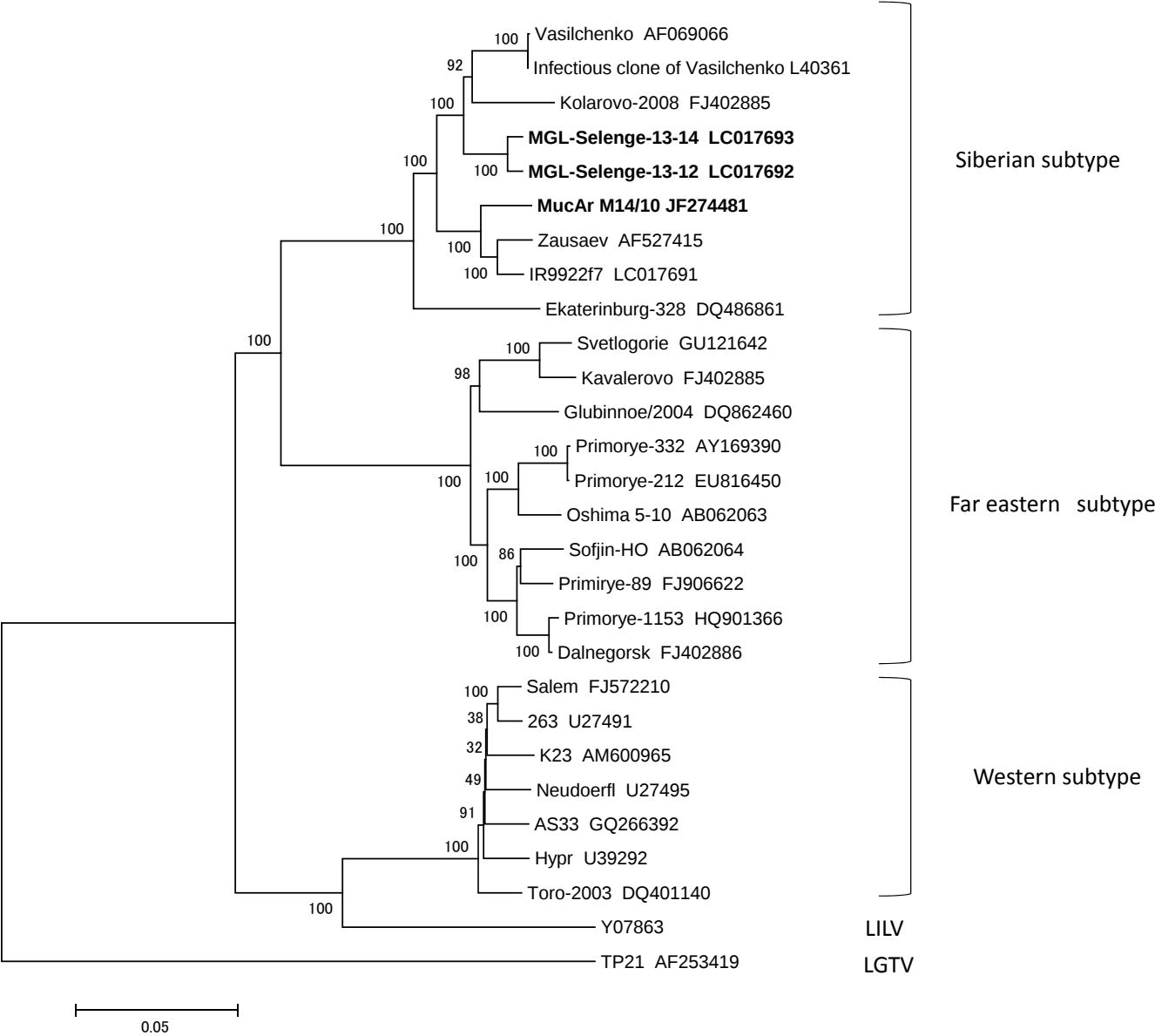
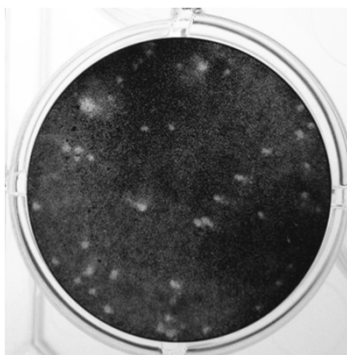


Fig. 4-A

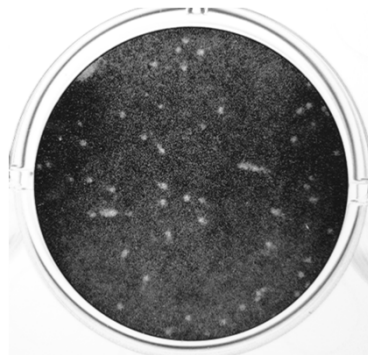


Fig. 4-B

MGL-Selenge-13-12



MGL-Selenge-13-14



IR99 2f7

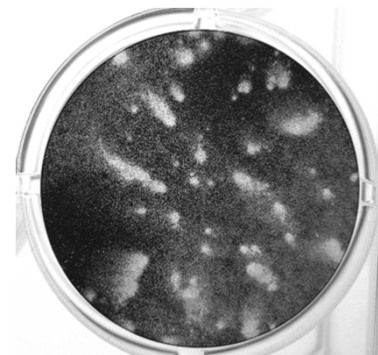


Fig. 5

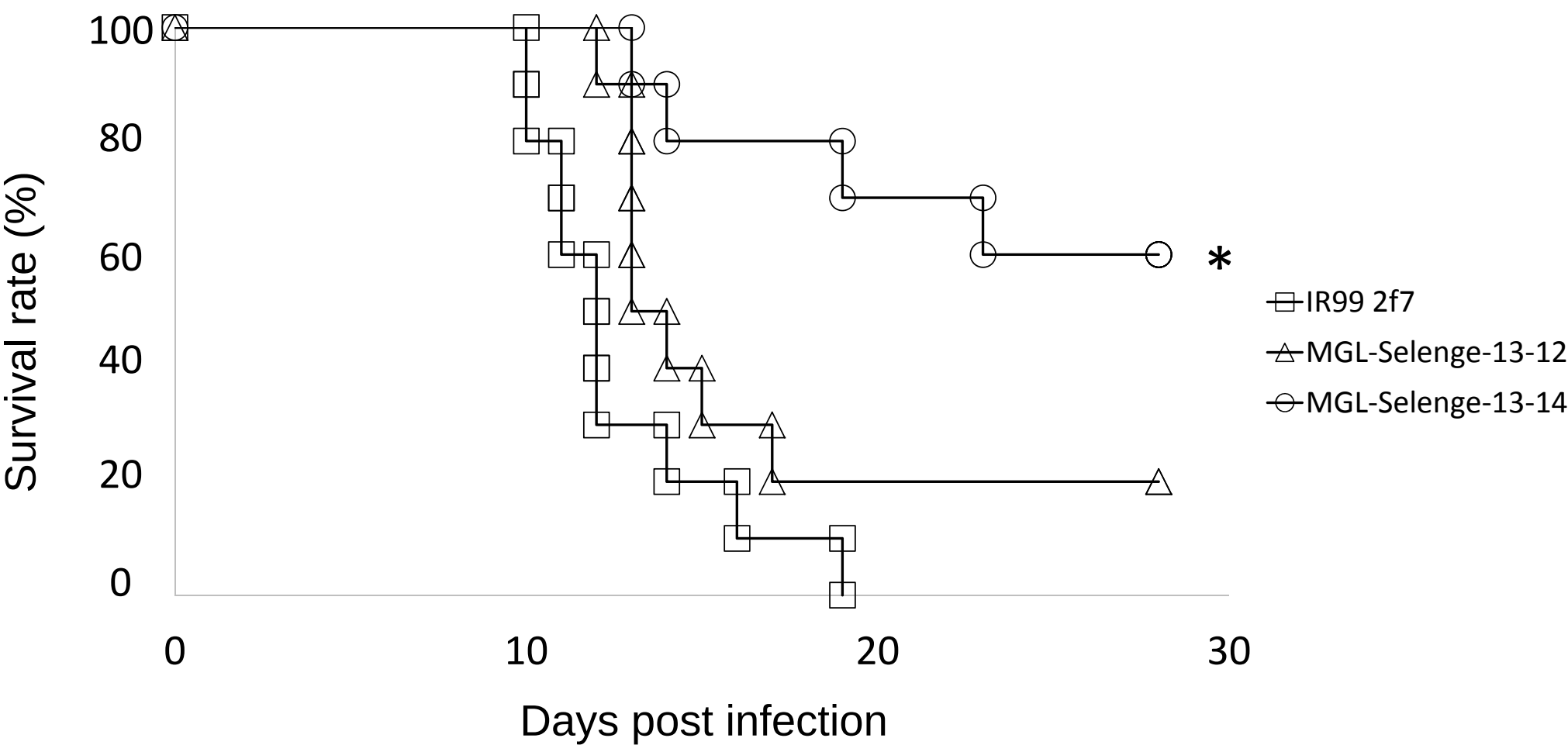


Fig. 6

