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

BHK	baby hamster kidney
CPE	cytopathic effect
p.i.	post infection
E	envelope
IFA	immunofluorescence assay
LGTV	Langat virus
MOI	multiplicity of infection
ORF	open reading frame
PCR	polymerase chain reaction
PFU	plaque forming unit
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
TBE	tick-borne encephalitis
TBEV	tick-borne encephalitis virus
UTR	untranslated region

Abstract

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

Keywords: Flavivirus, Tick-borne encephalitis, Mongolia

Introduction

Tick-borne encephalitis virus (TBEV), a member of the genus *Flavivirus* within the *Flavivirdae* 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

81 subtype, respectively (Frey et al., 2012; Khasnatinov et al., 2010). However, minimal data are

82 available concerning the biological characteristics of Mongolian TBEV strains (e.g., virulence and

83 viral multiplication).

84   In this study, we collected ticks (*Ixodes persulcatus*) from Selenge aimag in Mongolia, and

85 isolated the TBEV. We detected the TBEV antigens and genomic ribonucleic acid (RNA) in cell

86 cultures inoculated with tick homogenates. Sequencing revealed that the isolated viruses belonged to

87 the Siberian subtype of TBEV. Viral growth and plaque morphology were assessed and the

88 pathogenicity of the viral isolates was analyzed in a mouse model.

89

90 **Materials and Methods**

91

92 **Tick collection and virus isolation**

93   TBEV strains were isolated from ticks (*I. persulcatus)* collected in Bugant village, Selenge

94 aimag, in northern Mongolia, in 2012 (Fig. 1). In total, 680 ticks were collected by dragging flannel

95 sheets over the vegetation and pooled into groups of 20-30 ticks. The pools were washed with

96 ethanol and homogenized in phosphate buffered saline with a pestle. Each homogenized suspension

97 was centrifuged, and the supernatant was collected and stored at –80°C until the inoculation step.

98   Baby hamster kidney (BHK) cells were grown in 24-well plates. Then inoculated with the

99 supernatants collected in the previous step, and incubated at 37°C under 5% CO_2 for 1 h. After 2–4

100 days, the cells were checked for cytopathic effect (CPE) and supernatants from cells showing a CPE

101 were harvested and stored at –80°C. The viruses in these samples were identified by

102 immunofluorescence assay (IFA) using anti-tick borne flavivirus antibodies and reverse transcription

103 polymerase chain reaction (RT-PCR). All stock viruses were propagated once in BHK cells.

104

105 **Detection of viral antigens**

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

107   incubation at 37°C under 5% CO_2, the cells were fixed with 4% paraformaldehyde and permeabilized

108   with 0.1% Triton X-100. After blocking with 2% bovine serum albumin, the cells were incubated with

109   polyclonal hyper-immune murine ascites fluid from Langat virus (LGTV) infected mice (which is

110   cross-reactive to TBEV), followed by Alexa 555-conjugated anti-mouse immunoglobulin G

111   antibodies (Invitrogen, Carlsbad, CA).

112

113   **RT-PCR**

114   Viral RNA was extracted from BHK cells using ISOGEN (Nippon Gene, Tokyo, Japan) and

115   reverse-transcribed with random primers using M-MLV Reverse Transcriptase (Life Technologies,

116   Carlsbad, CA). The TBEV-specific sequence was amplified with Platinum *Taq* polymerase

117   (Invitrogen). To amplify the envelope (E) protein gene of TBEV, universal primers for the Far-Eastern

118   and the Siberian subtypes of TBEV were designed and used. (Mongolia-F:

119   5'-GGTYATGGARGTYRCRTTCTCTCG-3', and Mongolia-R:

120   5'-TCCCAGGCGTGYTCTCCKATCACTGT-3').

121

122   **TBE viral gene sequencing**

123   The nucleic acid sequences of the viral genomes were determined by direct sequencing. The

124   cycle sequencing reactions were performed using a BigDye™ Terminator Cycle Sequencing Kit (Life

125   Technologies), and the sequences were determined with a 3130 Genetic Analyzer (Life Technologies).

126   The primers used for sequencing were shown in Supplementary Table 1.

127

128   **Phylogenetic analysis**

129   A phylogenetic analysis was performed using the complete E gene and the complete full

130   sequence genomes of the TBEV strains. LGTV was used as the outgroup. Genetyx version 8 was

131   used to generate the multiple alignments. MEGA 6 (http://www.megasoftware.net/) was used to

132   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% CO_2.

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 10^3 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

211   strains were inoculated into mice. Viral multiplication in MGL-Selenge-13-12-infected mice sera

212   were observed from 3 days p.i., but not in MGL-Selenge-13-14-infected mice sera (Fig. 6).The virus

213   was detected in the brain from 9 days p.i. in all MGL-Selenge-13-12-infected mice and only one

214   mice infected with MGL-Selenge-13-14 at 9 and 11 days p.i. respectively. The virus titer reached 8.9

215    $\times 10^7$ PFU/ml at 11 days p.i. in the mice inoculated with MGL-Selenge-13-12 and was significantly

216   higher than that in the mice infected with the MGL-Selenge-13-14 (Fig. 6).

217

218

219   **Discussion**

220

221   In this study, nine TBEVs (MGL-Selenge-13) were isolated from *I. persulcatus* collected in

222   Bugant village, Selenge aimag, Mongolia (Figs. 1, and 2). The TBEV detection rate in ticks (1.3%)

223   was similar to that in a previous study (1.6%) in Mongolia (Frey et al., 2012). TBEV infected human

224   cases were found mainly in northern Mongolia, especially Selenge aimag (Walder et al., 2006). The

225   Siberian subtype (92M and MucAr M14/10) of TBEV was also detected from a patient and ticks in

226   northern Mongolia (Frey et al., 2012; Khasnatinov et al., 2010). These results showed that the

227   Siberian subtype of TBEV is endemic in northern Mongolia. This area is located next to the Asian

228   part of Russia, which is known to be a severe TBEV endemic region (Hayasaka et al., 2001). Human

229   activity may have contributed to the transmission of TBEV from Siberian Russia to Mongolia via the

230   Trans-Siberian railway (Frey et al., 2012; Kovalev et al., 2009). Additionally, Mongolia is an

231   important place for wild bird migration. There are transmission directions of influenza A virus

232   between Russia and Mongolia via birds migration (Kang et al., 2011). Surveillance in Russia

233   showed wild birds were bitten by ticks infected with TBEV (Mikryukova et al., 2014). It is possible

234   that wild birds may contribute to the transmission of TBEV into Mongolia.

235   From our phylogenetic analysis (Fig. 3-A), the MGL-Selenge-13 strains were classified in

236   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

291 **Acknowledgements**

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298

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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

		MGL-Selenge-13-12	MGL-Selenge-13-14	McuAr M14/10	92M	IR99 2f7
	Amino acid					
Nucleotide	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.
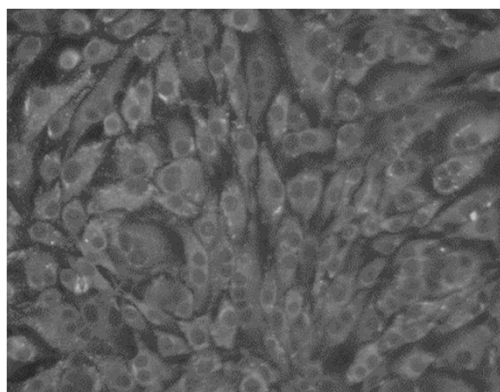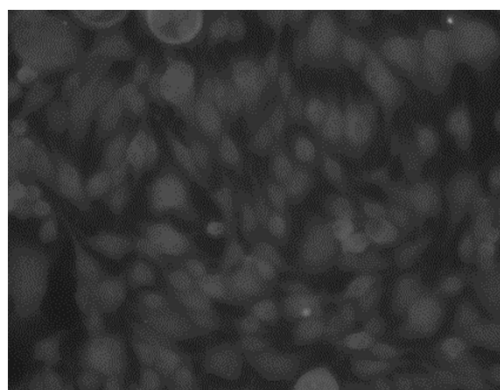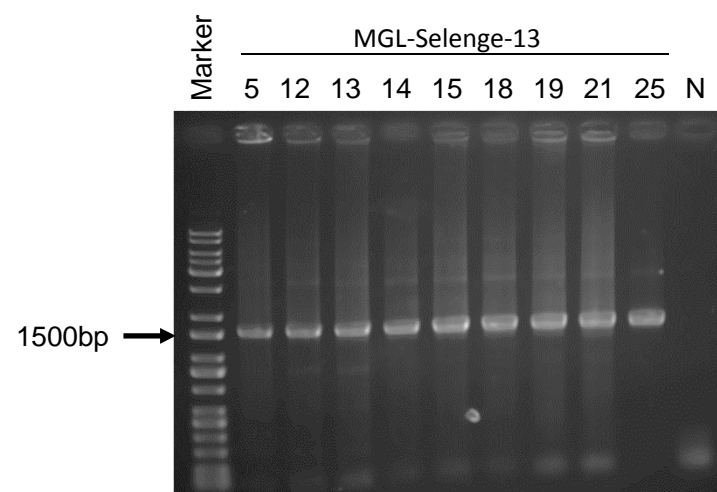
Fig. 3-A

E protein gene (1,488 nt)
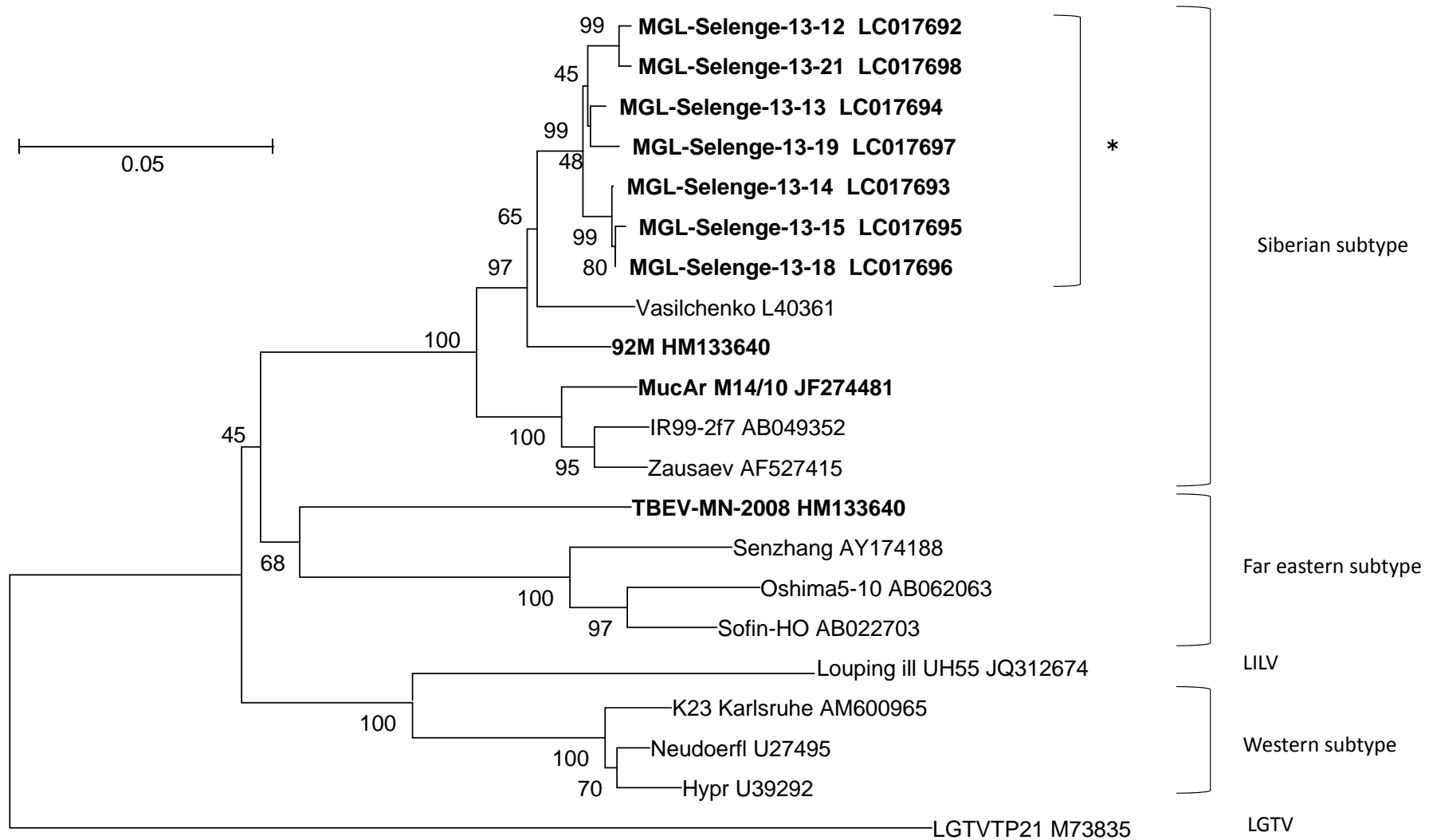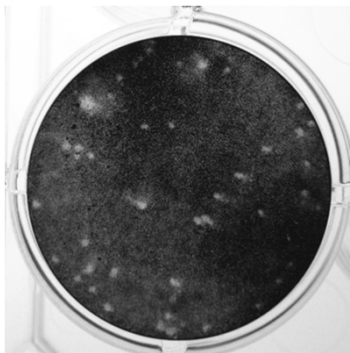
Fig. 3-B

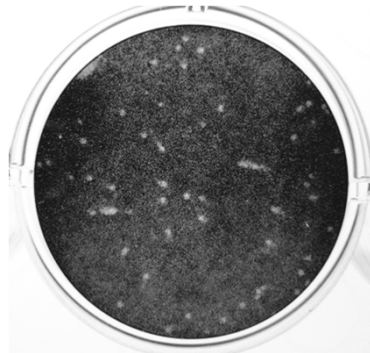Complete ORF (11,106 nt)
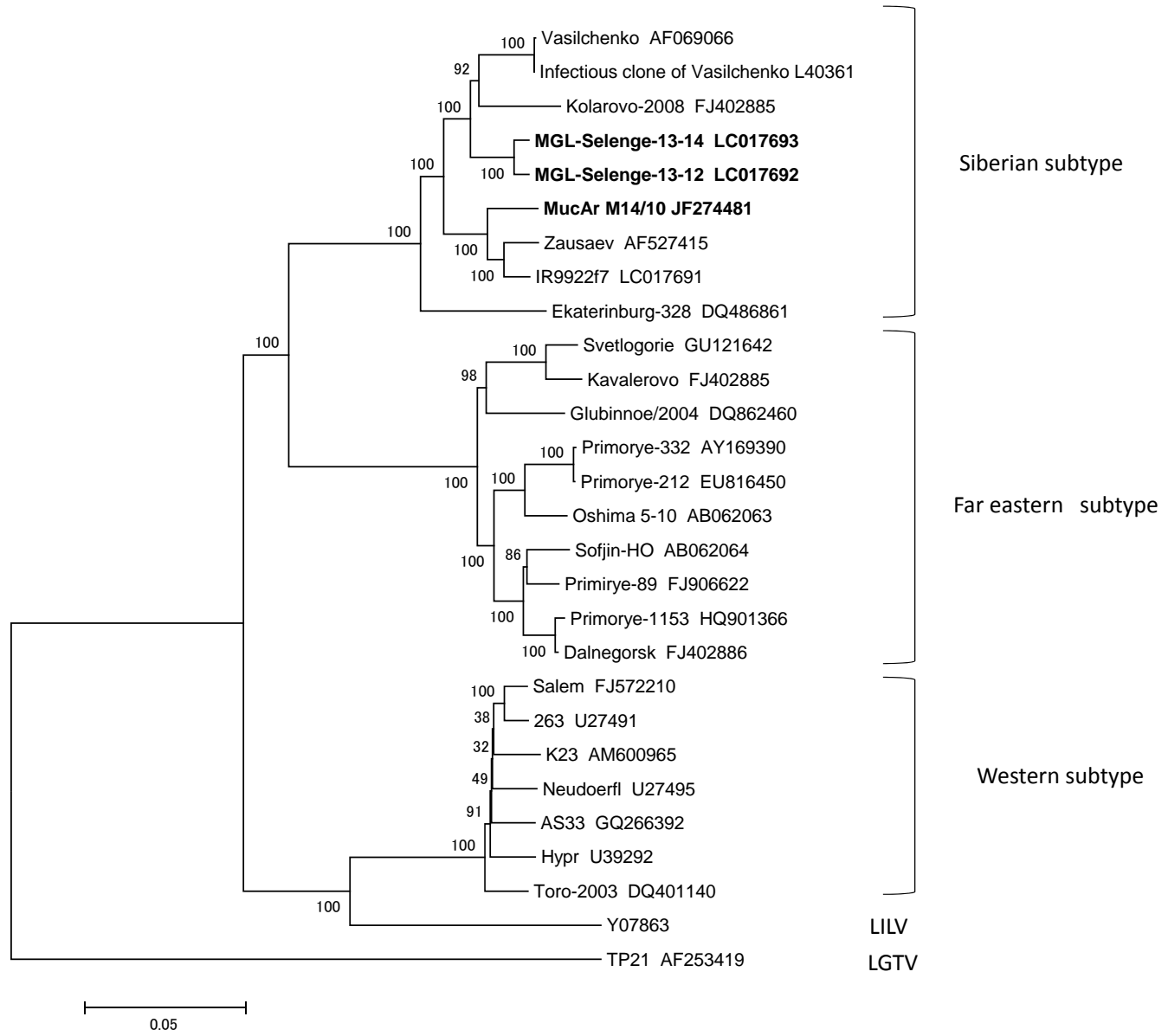
Fig. 4-A
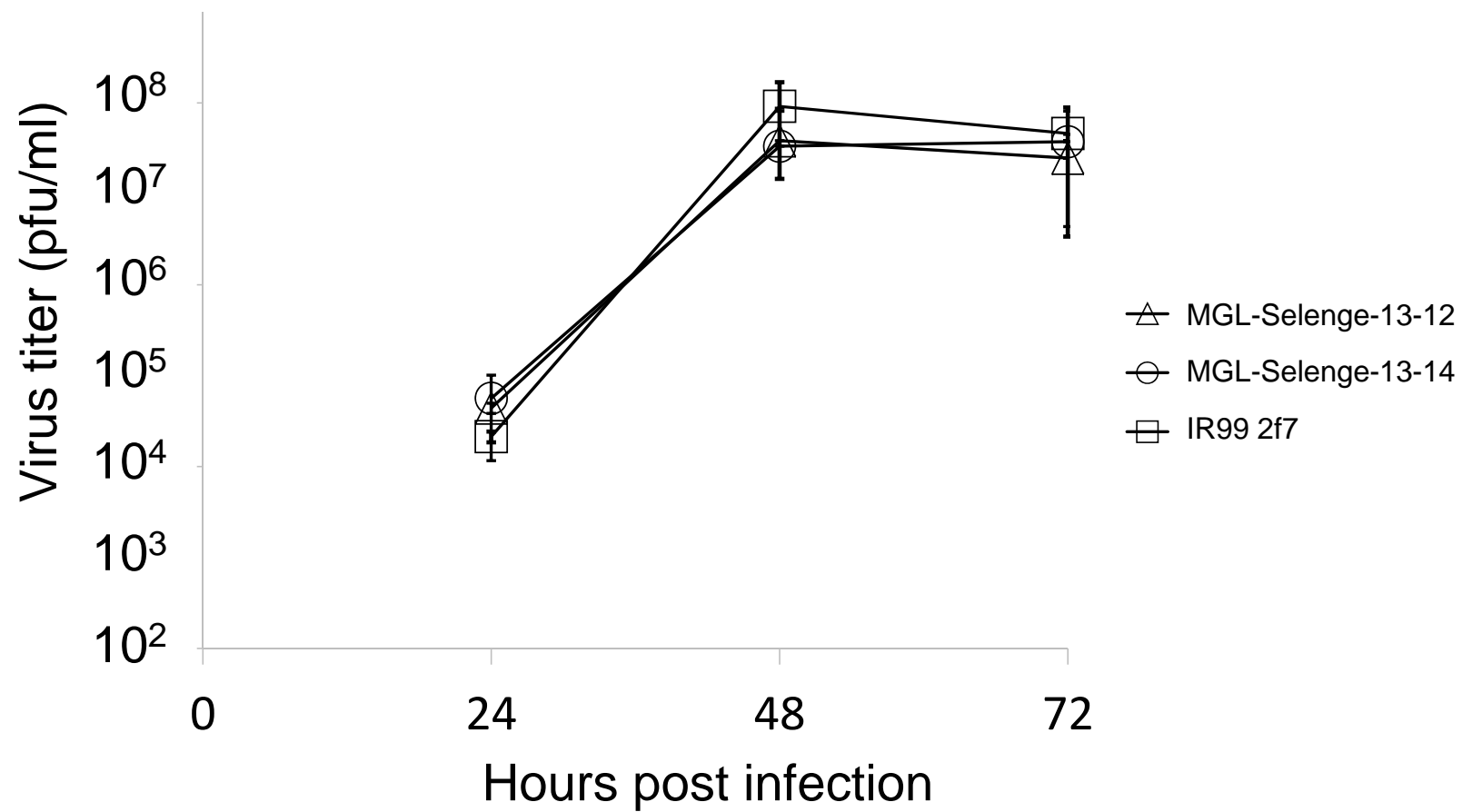
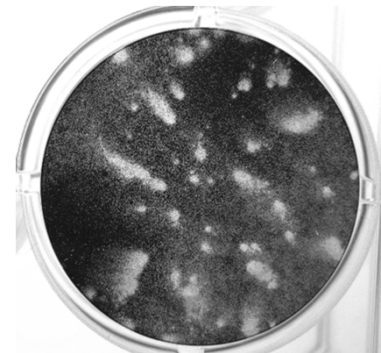Fig. 4-B

MGL-Selenge-13-12   MGL-Selenge-13-14   IR99 2f7
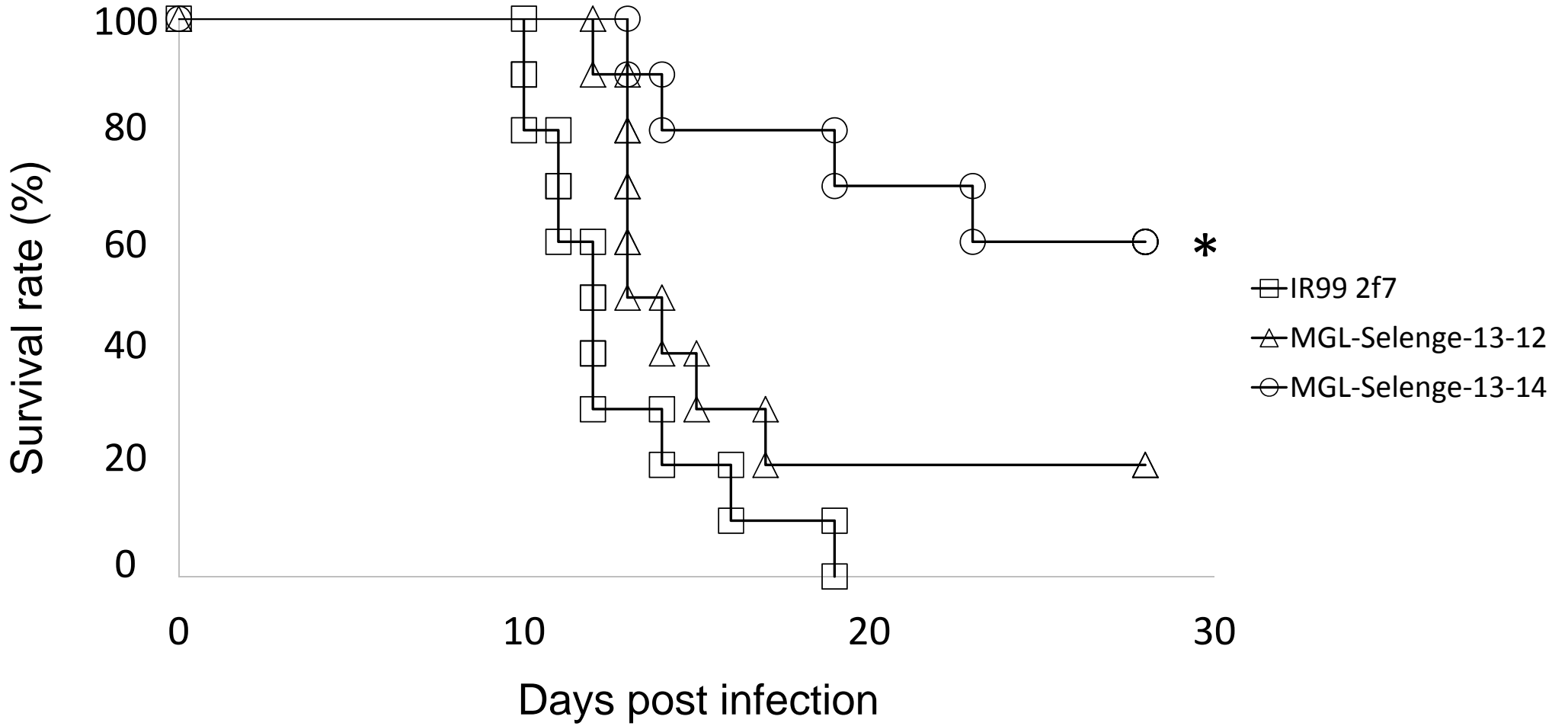
Fig. 5

Fig. 6