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

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Word count for Abstract: 165
Word count for text: 2746
<table>
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<tr>
<th>Abbreviation</th>
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<td>BHK</td>
<td>baby hamster kidney</td>
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<tr>
<td>CPE</td>
<td>cytopathic effect</td>
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<td>p.i.</td>
<td>post infection</td>
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<td>E</td>
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<td>immunofluorescence assay</td>
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<td>LGTV</td>
<td>Langat virus</td>
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<td>multiplicity of infection</td>
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<td>TBEV</td>
<td>tick-borne encephalitis virus</td>
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<td>UTR</td>
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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 *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% CO₂ 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’-TCCCAGGCCTGYTCTCCKATCAGCTG-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
evaluated using 500 bootstrap replicates. The GenBank Accession Numbers of the sequences were shown in Fig. 3.

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

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

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

**Animal model**

Each virus was inoculated subcutaneously at 10³ PFU into ten 5-week-old female C57BL/6J mice (Japan SLC, Shizuoka, Japan). Surviving mice were monitored for 28 days p.i. to determine survival curves and mortality rates. Onsets of disease were estimated at 10% weight loss compared with the weight before virus infection. For the analysis of viral distribution in tissues, three to four mice were sacrificed on 3, 6, 9, 11 days p.i., and sera and brains were collected. Organs were individually weighted, homogenized, and prepared as 10% (w/v) suspensions in phosphate buffered saline with 10% fetal bovine serum. Suspensions were then clarified by 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.
Experimental protocols were approved by the Animal Care and Use Committee of Hokkaido University.

Results

Isolation and identification of TBEV in Mongolia

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

Genetic analysis of the isolated TBEV strains

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

The nucleotide and amino acid sequence of the viral E gene were compared with MGL-Selenge-13-12, MGL-Selenge-13-14, MucAr M14/10 (isolated in Mongolia), M92 (isolated in Mongolia), and IR99 2f7 (isolated in Russia) (Table 1). All strains highly homologous (>90%) in both their nucleotide and amino acid sequences. The full length sequences of MGL-Selenge-13-12 and MGL-Selenge-13-14 (11106 nt) were also compared. The nucleotide homology was 99.1% (11005 nt /11106 nt) in the complete sequences, including the 5’- and 3’- untranslated regions.
Nucleotide substitutions were observed in the 5’-UTR (one nucleotide) and 3’-UTR (seven nucleotides), but no deletions or insertions were observed in these regions. The amino acid differences were located only in the viral E (residues 580, 597, and 631), NS3 (residues 1743, 1992, and 2046), and NS5 (residues 2623, 3221, 3223, 3352, 3357, 3403, and 3409) genes (Table 2).

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

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

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

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 mouse sera were observed from 3 days p.i., but not in MGL-Selenge-13-14-infected mouse 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 mouse infected with MGL-Selenge-13-14 at 9 and 11 days p.i. respectively. The virus titer reached $8.9 \times 10^7$ 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
TBEV, the strains diverged into two subclusters. The MGL-Selenge-13 strains were classified in the same subcluster as the 92M strain but not the MucAr M14/10 strain. The MucAr M14/10 strain was in a subcluster with the IR99 2t7 strain isolated from Irkutsk (Hayasaka et al., 2001). It has been reported that several subclusters of Siberian TBEV are endemic in Russia (Hayasaka et al., 2009). Our results suggest that at least two subclusters of TBEV invaded from Russia into Mongolia, independently.

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

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

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

In summary, we newly isolated the Siberian subtype of TBEV in Selenge aimag in Mongolia. Several strains showed different levels of virulence in a mouse model, indicating that a few naturally occurring mutations affect the virulence of the endemic strains in Mongolia. Minimal data are available about TBEV, which is endemic in Mongolia due to the lack of established diagnostic systems for TBE. Moreover, the Far-Eastern subtype of TBEV was also detected in other survey in Mongolia (Khasnatinov et al., 2010). To determine the distribution of TBEV in Mongolia, additional
epidemiological studies are necessary. Our results could be an important platform for monitoring TBEV to evaluate the epidemiological risk in TBE endemic areas of Mongolia.

Acknowledgements

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References


Figure legends

Fig. 1. Geographical distribution of the study area.

Fig. 2. Detection of TBEV specific antigens and RNA. Immunofluorescence analysis of BHK cells.
BHK cells were inoculated with the supernatant from TBEV positive tick homogenates (MGL-Selenge-13-12) (A) or a mock sample (B) and TBEV antigen was detected with anti-tick borne flavivirus antibodies. (C) The E protein gene of TBEV (1488 bp) were amplified by RT-PCR from cells inoculated with TBEV positive tick homogenates (MGL-Selenge-13-5, -12, -13, -14, -15, -18, -19, -21, and 25) and mock treated cells (N).

Fig. 3. (A) Phylogenetic tree of TBEV strains based on 1488 nucleotides of the viral E gene. LGTV was used as an outgroup. The percentage of bootstrap values are shown next to the branches. *Isolated TBEV strains in this study. Accession numbers are shown after the virus strains. (B) Phylogenetic tree of TBEV strains based on the ORF of the viral gene. LGTV was used as an outgroup. Bold letters indicate the strains isolated in Mongolia.

Fig. 4. (A) Comparison of the growth curves of IR99 2f7, MGL-Selenge-13-12, and MGL-Selenge-13-14.
A monolayer of BHK cells was infected with each virus at a multiplicity of infection (MOI) of 0.01. At each time point, the medium was harvested and virus titers were determined using a plaque assay in BHK cells. (B) Plaque morphology of IR99 2f7, MGL-Selenge-13-12, and MGL-Selenge-13-14 in BHK cells. BHK cells were stained with crystal violet (0.1%) at 96h p.i.

Fig. 5. Survival of mice inoculated with TBE viral strains IR99-2f7, MGL-Selenge-13-12, and MGL-Selenge-13-14. Mice were inoculated subcutaneously with $10^3$ PFU of each virus and monitored for 28 days. * MGL-Selenge-13-14 showed significant differences in survival rate compared with IR99 2f7 and MGL-Selenge-13-12 ($P<0.05$). Survival rates were calculated using the Kaplan-Meier method and $P$-values for the differences in survival rates were calculated using
Fig. 6 Viral multiplication in mice organs. Mice were inoculated subcutaneously with $10^3$ PFU of each virus. Virus titers in the blood and the brain at the indicated days after infection were determined by plaque assays. The limits of virus detection for the assay was $10^2$ PFU/ml. Error bars represent the standard deviation (n=3 or 4). Asterisks indicate significant differences compared with 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

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<th>MGL-Selenge-13-12</th>
<th>MGL-Selenge-13-14</th>
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<th>M14/10</th>
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<td>MGL-Selenge-13-12</td>
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Table 2. Amino acid differences between MGL-Selenge-13-12 and MGL-Selenge-13-14

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**Table 3. Mortality and morbidity of the virus infected mice**

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<tbody>
<tr>
<td>IR99 2f7</td>
<td>100 (10/10)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100 (10/10)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.4 ± 1.8</td>
<td>12.7 ± 2.9</td>
</tr>
<tr>
<td>MGL-Selenge-13-12</td>
<td>100 (10/10)</td>
<td>80 (8/10)</td>
<td>10.3 ± 1.5</td>
<td>13.8 ± 1.6</td>
</tr>
<tr>
<td>MGL-Selenge-13-14</td>
<td>50 (5/10)</td>
<td>40 (4/10)</td>
<td>14.0 ± 4.3</td>
<td>17.3 ± 4.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Morbidity of mice was estimated by >10% of weight loss.

<sup>b</sup>Number of sick mice / number of infected mice.

<sup>c</sup>Number of dead mice / number of infected mice.
Fig. 2.

A

B

C

MGL-Selenge-13

<table>
<thead>
<tr>
<th>Marker</th>
<th>5</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>18</th>
<th>19</th>
<th>21</th>
<th>25</th>
<th>N</th>
</tr>
</thead>
</table>

1500bp
Fig. 3-A

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

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

Virus titer (pfu/ml)

Hours post infection

- MGL-Selenge-13-12
- MGL-Selenge-13-14
- IR99 2f7
Fig. 4-B

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

Survival rate (%) vs. Days post infection for different groups:
- IR99 2f7
- MGL-Selenge-13-12
- MGL-Selenge-13-14

* indicates significant difference.
Fig. 6

**Virus titer (Log_{10} pfu/ml)**

**Days post infection**

**Blood**

**Brain**

**MGL-Selenge-13-12**

**MGL-Selenge-13-14**