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ORIGINAL ARTICLE

# Characteristics of far eastern strains of tick-borne encephalitis virus

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**Abstract** A comparative study of biological, molecular and genetic characteristics of a collection of ten strains of tick-borne encephalitis virus (TBEV) isolated in Primorsky Krai before 1960 and stored in a lyophilized state for a prolonged period (over 65 years) is presented. The collection includes the Sofjin strain isolated from the brain of a fatal case in Primorsky Krai in 1937 and transferred to the Scientific Research Institute of Epidemiology and Microbiology (Vladivostok) in 1953. All lyophilized viral strains demonstrated great preservation and high infectious activity in the model of 2-day-old non-inbred mice. Whole-genome sequencing showed that all strains belong to the Far East TBEV subtype, comprising three clusters of Sofjin-, Oshima- and Senzhang-like strains. We show that SofjinPYB, Sofjin (Vector) and Sofjin-HO strains form a separate branch of the phylogenetic tree and are closely related to Khabarovsk-Obor-4, but not to the original Sofjin strain. The Sofjin-1953, Sofijin-Chumakov, SofjinKSY and SofjinCDC strains are genetically close to each other and can be used as reference strains for comparative analysis of the tick-borne encephalitis virus population.

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## Introduction

Tick-borne encephalitis virus (TBEV) was discovered and described in the Far East in 1937 by two medical expeditions led by L. A. Zilber. Two teams simultaneously carried out comprehensive studies in the Khabarovsk (northern team) and Primorsky (southern team) districts. Twenty-six strains were isolated during 3 months of work, of which 18 were isolated from clinical (including fatal) cases of the previously unknown neuroinfection. Next, a comparative study of the biological characteristics of isolates from the Khabarovsk and Primorye territories was performed. At that time, the authors were unable to identify differences between these groups of strains [1]. Among these was the Sofjin strain, isolated by the southern team from the brain of a fatal case. After repeated passaging in white mice, the Sofjin strain showed stable biological properties. It became a classic prototype strain and was used for the development of tick-borne encephalitis (TBE) vaccines.

Subsequently, thousands of TBEV strains were isolated in the vast territory of the Eurasian continent. All TBEV strains were grouped based on their biological and molecular-genetic features into three main subtypes: Far Eastern, European, and Siberian [2].

In our previous work, using biological characteristics and genome sequencing, we have shown that 36 TBEV strains isolated from fatal and clinical cases in the Primorye Territory belong to a single Far East subtype and can be divided into three clusters [3, 4]. Here, we compare the isolates to the older strains of TBEV to characterize the Far Eastern TBEV subtype. This study describes a comparative analysis of biological, molecular and genetic characteristics of 10 strains isolated before 1960, including the classical prototype strain Sofjin, which had been stored in a lyophilized state since 1953.

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## Materials and methods

### Tick-borne encephalitis virus strains

Ten TBEV strains isolated in Primorsky Krai and lyophilized in 1960 were used for this study. The sealed glass vials with TBEV were stored at -20 °C. The strain collection was created by L.G. Tatarinova, the first head of TBE laboratory of the Scientific Research Institute of Epidemiology and Microbiology (Vladivostok). This collection included four strains isolated from brains of fatal cases: two strains from patients' blood, two strains from *Ixodes persulcatus*, one strain from *Haemaphysalis japonica*, and one strain from mouse brain (*Apodemus peninsulae*). The set of viruses also included the lyophilized Sofjin strain, isolated in Primorsky Krai in 1937 [5] and submitted by E.N. Levkovich, the head of TBE laboratory of the Ivanovsky Institute of Virology of the USSR Academy of Medical Sciences, to the TBE laboratory of the Scientific Research Institute of Epidemiology and Microbiology (Vladivostok) to carry out virological studies in 1953. The list of strains from various areas of Primorsky Krai used in this study is presented in Table 1.

### Methods for virological studies

Lyophilized samples were diluted in 1 ml of the culture medium 199 (Chumakov Institute of Poliomyelitis and Viral Encephalitides) and placed in a refrigerator for one day at +4 °C. The pathogens were isolated by intracerebral infection of 2-day-old non-inbred mice.

Neurovirulent and neuroinvasive properties of 10 strains of the Far East subtype of TBEV were studied. Strains of 1 to 2 passages in 2-day outbred ICR/CD-1 mice (Pushchino) were used for the study. Titers of the TBEV strains were determined by intracerebral (i/c) and subcutaneous (s/c) injections of mice weighing 10 to 12 g with 0.03 ml and 0.2 ml of inoculum, respectively. The virus titer was calculated by the Reed and Muench method [6]. The invasiveness index (I.I.) was calculated as the difference in infectious titer for s/c and i/c infection.

Comparative analysis of the neurovirulence and neuroinvasive properties of these strains was carried out using i/c (0.03 ml) and s/c (0.2 ml) infections of mice weighing 14 to 16 g at a single dosage of 100 $LD_{50}$ of virus, which was determined by intracerebral titration mice weighing 10-12 g. Ten mice were used at each test point. The duration of observation was 21 days. The assessment criteria for neurovirulence properties were the percentage of surviving animals and their average survival time (AST).

The titers of TBEV strains were determined by plaque assay in PK cell culture and expressed as as plaque-forming units (PFU). For this purpose, one-day PK cell monolayers grown in 24-well plates were infected with tenfold dilutions of TBEV strains. After 1 hour of contact at 37 °C, the infected monolayer was washed three times with cell culture medium 199 and then overlaid with maintenance medium containing 0.6% carboxymethylcellulose solution (manufactured by ICN Biomedical, Inc) in 199 medium supplemented with 1% bovine fetal serum. Counting of plaques was carried out after 4–5 days after Amido Black 10B coating, and the virus titer was expressed as PFU/ml.

### Methods for molecular genetic studies

#### *Viral RNA extraction and cDNA synthesis*

Total RNA was extracted from 100 μL of a 10% suspension of brain or plasma in phosphate-buffered saline (PBS) using TRIzol Reagent (Life Science, USA) in accordance with the manufacturer's recommendations. Then, the RNA was precipitated, washed twice with 1 ml of 80% ethanol

**Table 1** The tick-borne encephalitis virus strains that had been isolated in Primorsky Krai before 1960

| No | Strain | Year of isolation | Source of isolation | Region of isolation | Passage of the strains before freeze-drying |
|---|---|---|---|---|---|
| 1 | Sofjin-1953 | 1937 | The brain dead patient, strain transferred to the laboratory in 1953 | Primorsky Krai | unknown |
| 2 | Golubnichiy | 1958 | The brain dead patient on the 5th day of illness | Michailovsky | 2 |
| 3 | Primorye-1035 (P-1035) | 1958 | The brain dead patient on the 4th day of illness | Ussuryisky | 2 |
| 4 | Primorye-696 (P-696) | 1960 | The brain dead patient on the 8th day of illness | Daĺnerechensky | 2 |
| 5 | Primorye-1285 (P-1285) | 1958 | The blood on day 5 of disease | Shkotovsky | 3 |
| 6 | Primorye-1284 (P-1284) | 1958 | The blood on day 9 of disease | Shkotovsky | 3 |
| 7 | Primorye-1056 (P-1056) | 1958 | *Ixodes persulcatus* | Daĺnerechensky | 2 |
| 8 | Primorye-949 (P-949) | 1958 | *Haemaphysalis japonica* | Daĺnerechensky | 3 |
| 9 | Primorye-1001 (P-1001) | 1958 | *Ixodes persulcatus* | Suputinsky Park | 2 |
| 10 | Primorye-512 (P-512) | 1959 | *Apodemus peninsulae* | Suputinsky Park | 3 |

and air-dried for 5 min. The RNA precipitate was suspended in 50 μL of Milli-Q water and used as a template in the reverse transcription (RT) reaction as soon as possible. To obtain cDNA using RT, a mixture of 5 μL of RNA, 50 pM hexanucleotide primers, 200 mM each NTP, 5 mM $MgCl_2$, 200 units of MMLV reverse transcriptase (Promega, Madison, WI) and Milli-Q water (to a total volume of 50 μL) was incubated at 42 °C for 30 min. cDNA samples designated for transport were precipitated by adding an equal volume of isopropanol and stored as an alcohol suspension.

*Amplification*

We have previously described oligonucleotide primers for PCR that were based on published sequences of the Sofjin-HO, Senzhang, 205, Glubinnoe and Aina strains for the amplification of 38 overlapping fragments of sense and antisense DNA strands, with the average length of amplified fragments being about 1000 base pairs [4]. For the purpose of amplifying each fragment, a mixture of 0.5 μL of cDNA, 50 pM of the relevant sense and antisense PCR primers, 25 μl of 2x PCR mixture (Fermentas, Lithuania) and Milli-Q water (to a total volume of 50 μL) was incubated at 95 °C for 1 min and then used in the cycle sequencing reaction (25 cycles at 96 °C for 5 s, 57 °C for 5 s, and 72 °C for 30 s). Amplicons were analyzed by electrophoresis in a 0.75% agarose gel in 1x TEA (triethanolamine) buffer with ethidium bromide. DNA bands were cut from the gel under UV light, and the DNA was eluted from the gel using the freeze-thawing method. The concentration of eluted DNA was determined using Nano View (GE Healthcare, USA).

*PCR fragment sequencing*

Each sequencing reaction mixture contained about 50 to 100 ng of purified double-stranded DNA fragments, 3.2 pmol of primer and reaction mixture containing four fluorescein-labeled dideoxynucleotide terminators (BigDye V 3.1, Life Science, USA). The cycle sequencing parameters used were as described in the manufacturer's protocol (25 cycles of 96 °C for 30 s, 50 °C for 60 s and 60 °C for 4 min). Reaction products were precipitated by ethanol-acetate as described in the manufacturer's recommendations. The pellet was resuspended in 16 μL of reaction inhibitor, heated at 95 °C for 2 min and kept on ice before being loaded into an Applied Biosystems Prism 3100 sequencer.

All sequences were deposited in the GenBank database (Golubnichiy, KU761567; Primorye-512, KU761568; Primorye-696, KU761569; Primorye-949, KU761570; Primorye-1001, KU761571; Primorye-1035, KU761572; Primorye-1056, KU761573; Primorye-1284, KU761574; Primorye-1285, KU761575; Sofjin-1953, KU761576).

*Phylogenetic analysis*

Genome assembly and alignment were performed using Bioedit software (available from http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic analysis was carried out using MEGA v.5.0 software [7]. Evolutionary distances were assessed by the maximum-likelihood method using the two-parameter model [8]. FigTree software (available from http://tree.bio.ed.ac.uk/software/figtree) was used for graphical representation of the phylogenetic trees.

## Results

### Biological characteristic of the strains

First, it was necessary to examine the viability of strains that had been freeze-dried and stored for more than 65 years. It was found that nearly all of the strains caused disease in infected suckling mice at the first passage at different times after inoculation. For example, mice infected with the Sofjin-1953, Golubnichiy, Primorye-1035, Primorye-696, Primorye-1056, Primorye-949 and Primorye-1001 strains came down with disease on days 5 and 6 (Table 2). Specific clinical signs of infection in 2-day-old suckling mice infected with different strains were the same: hunched posture, significant tremor, and loss of appetite (absence of milk in the stomach).

Virus preparations from the first passage, and occasionally from the second passage, were used for further analysis of biological, molecular and genetic characteristics. All strains had high titers after i/c infection of non-inbred mice except for the Primorye-1284 strain, which was isolated from a patient's blood on the ninth day of disease. This strain had a low titer after s/c infection (4.5 log $LD_{50}$) (Table 3). Most strains actively grew in PK cell culture to a titer of $10^9$ log $PFU_{50}$/ml. Only the strains Primorye-949 and Primorye-1284 accumulated in culture medium at lower levels ($10^4$–$10^5$ log $PFU_{50}$/ml).

The neurovirulence and neuroinvasiveness of these strains represent an important characteristic of TBEV. These strains demonstrated high neurovirulence (Fig. 1a) and various degrees of neuroinvasiveness (Fig 1b). High neurovirulence was demonstrated for the Sofjin-1953 strain, which quickly caused death of the animals after i/c infection, with an AST of 5.4 days and 11.0 days for s/c infection, with a survival rate of 0% (Table 3).

Another strain, Primorye-696, which caused the patient's death on day 8 after the onset of the disease, had

**Table 2** Activity of infectious process in 2-day mice infected with lyophilized strains of tick-borne encephalitis virus after 65-years storage. Note: The number of diseased mice depending on the day of observation for each strain is indicated

| No | Strain | The number of infected mice | Days disease mice | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 5 | 6 | 7 | 10 | 11 | 13 | 17 | 21 (Not diseased mouse) |
| 1 | Sofjin-1953 | 6 | 4 | | 2 | | | | | 0 |
| 2 | Golubnichiy | 7 | | 1 | 3 | 1 | | | | 2 |
| 3 | Primorye-1035 | 7 | 7 | | | | | | | 0 |
| 4 | Primorye-696 | 6 | 5 | | 1 | | | | | 0 |
| 5 | Primorye-1285 | 6 | | 1 | 1 | | 2 | | | 2 |
| 6 | Primorye-1284 | 7 | | | | 2 | The brain of 2 mice taken in the blind passage | | | 3 |
| 7 | Primorye-1056 | 10 | 8 | 2 | | | | | | 0 |
| 8 | Primorye-949 | 9 | 9 | | | | | | | 0 |
| 9 | Primorye-1001 | 6 | | 6 | | | | | | 0 |
| 10 | Primorye-512 | 6 | | | 1 | | | 1 | 4 | 0 |

**Table 3** Characteristics of biological activity of the strains of tick-borne encephalitis virus

| No | Strain | Titer of virus (lg $LD_{50}$/ml) | | Index of invasiveness (I.I.) | Virus titer in PK (lg $PFU_{50}$/ ml) | Survival rate (%) | | Average survival time of mice (days) | |
|---|---|---|---|---|---|---|---|---|---|
| | | i/c | s/c | | | i/c | s/c | i/c | s/c |
| 1 | Sofjin-1953 | 8,9 | 6,2 | 2,7 | $5,2 \times 10^9$ | 0 | 0 | 5,4 | 11,1 |
| 2 | Golubnichiy | 8,3 | 6,7 | 1,6 | $6,1 \times 10^9$ | 0 | 0 | 6,8 | 10,2 |
| 3 | P-1035 | 8,8 | 6,2 | 2,6 | $4,9 \times 10^9$ | 20 | 40 | 10,4 | 17,0 |
| 4 | P-696 | 10 | 8,3 | 1,7 | $2,1 \times 10^9$ | 0 | 20 | 8,2 | 11,8 |
| 5 | P-1285 | 9,5 | 7,1 | 2,4 | $2,5 \times 10^9$ | 30 | 50 | 12,5 | 14,8 |
| 6 | P-1284 | 8,8 | 4,5 | 4,3 | $1,7 \times 10^5$ | 10 | 30 | 8,1 | 12,4 |
| 7 | P-1056 | 7,9 | 5,2 | 2,7 | $3,2 \times 10^9$ | 0 | 50 | 7,3 | 16,4 |
| 8 | P-949 | 8,0 | 4,5 | 3,5 | $4,3 \times 10^4$ | 20 | 70 | 11,1 | 18,5 |
| 9 | P-1001 | 7,6 | 6,5 | 1,1 | $2,1 \times 10^9$ | 0 | 80 | 7,2 | 19,2 |
| 10 | P-512 | 6,7 | 4,7 | 2,0 | $2,4 \times 10^9$ | 10 | 40 | 8,3 | 14,7 |

AST values for i/c and s/c infection of 8.2 days and 11.8 days, respectively, with a survival rate of 0%. The Primorye-1284 strain demonstrated lower neuroinvasiveness, with an AST value for s/c infection of 12.2 days and a survival rate of 30%. A number of strains isolated from *Ixodes* ticks had higher survival rates after s/c infection: the AST value for the Primorye-1056 strain was 15.6 days, with a survival rate of 50%, while for the Primorye-1001 strain, it was 19.2 days, with a survival rate of 80%, although the survival rate after i/c infection was 0% and the AST values were 7.3 and 7.2 days, respectively.

## Molecular genetic characterization of the strains

Analysis of the molecular and genetic characteristics of these strains based on whole-genome sequencing showed that all of the strains belong to the Far Eastern TBEV subtype. Fig. 2 shows a phylogenetic tree in which the studied strains are located in three clusters: Sofjin-like (Primorye-1001, Primorye-696, Primorye-1035, Primorye-1285, Primorye-949), Oshima-like (Primorye-1284, Golubnichiy), and Senzhang-like (Primorye-512).

## Comparative molecular genetic characterization of Sofjin strains

We also carried out comparative molecular and genetic analysis of the whole genome of the Sofjin-1953 strain and other virus stocks currently known as Sofjin virus. As the phylogenetic tree shows (Fig. 3), the whole-genome sequences of six Sofjin-related strains are different. Three Sofjin strains (Sofjin-1953, SofjinKSY, Sofjin-Chumakov)

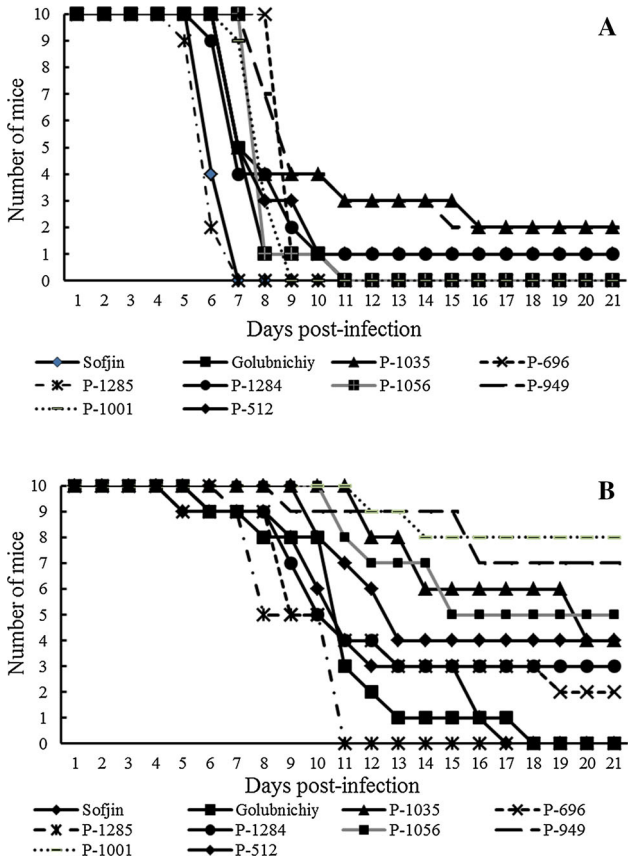


**Fig. 1** Neurovirulence and neuroinvasive properties of the strains of tick-borne encephalitis virus. Non-inbred mice were infected with a virus dose of 100 $LD_{50}$. **A** intracerebral injection (i/c, 0.03 ml); **B** subcutaneous injection (s/c, 0.2 ml)

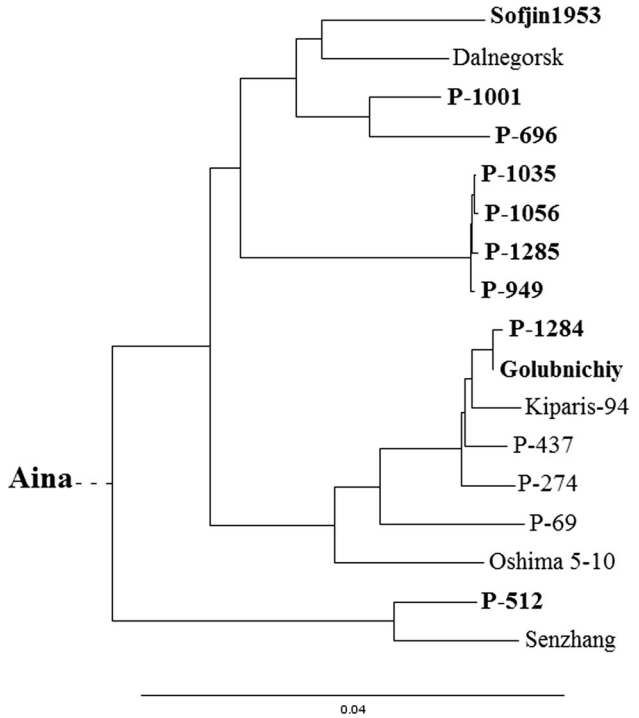


**Fig. 2** ML phylogenetic tree based on complete genome sequences of tick-borne encephalitis virus strains. Old strains are shown in bold. Aina is a typical Siberian strain

are located on a single branch and are virtually identical. Other Sofjin strains, including SofjinPYB, which was the first to be sequenced [9]; a Sofjin strain (accession no. JX498940) sequenced at the State Research Center of Virology and Biotechnology 'Vector', designated as Sofjin (Vector); and Sofjin-HO formed an independent branch of the phylogenetic tree. Comparative analysis of the sequences with the only published sequence of the E gene of strain Khabarovsk-Obor-4 showed that the SofjinPYB, Sofjin (Vector), and Sofjin-HO strains have an ancestor in common with the Obor strain, which was isolated by the northern team of the expedition. This analysis demonstrates that the Sofjin strains are divided into three groups: Khabarovsk-Obor-4 and Sofjin-HO; Sofjin-Chumakov, Sofjin-1953 and SofjinKSY; and Sofjin (Vector) and Sofjin-PYB (Fig. 3a).

In addition to the above strains, there is another Sofjin strain under the name of "Russian spring-summer encephalitis virus strain Sofjin" at the collection of the Special Pathogens Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention. There is a nucleotide sequence of the only fragment of the NS5 gene published in the GenBank database (AF013399) that we call SofjinCDC. We have conducted a comparative analysis of the NS5 gene sequences of the above-mentioned Sofjin-like strains to see how they are related to strain SofjinCDC and found that, compared to the SofjinCDC strain, SofjinKSY and Sofjin-1953 are closely related, as shown in the phylogenetic tree (Fig. 3b).

Table 4 shows amino acid substitutions in the E and NS5 proteins compared to the Sofjin-1953 strain. Table 4a demonstrates a complete accordance of amino acids in the E protein fragment in Khabarovsk-Obor-4 and Sofjin-HO strains. Other strains differ by the presence of at least one substitution. The presence of the specific substitutions N55D, H248Q and I351T allows two groups of strains to be identified, with common ancestor strains isolated by the southern and northern teams of the expedition. The southern group of strains includes Sofjin-1953, Sofjin-Chumakov and SofjinKSY; the northern group of strains is represented by Khabarovsk-Obor-4, Sofjin-HO, Sofjin (Vector) and Sofjin-PYB. The Senzhang and Oshima 5-10 strains probably have ancestors in common with the northern group of strains.

The sequence of the NS5 protein fragment is more conserved, and therefore the strains of Sofjin group have no significant differencesin this region, with the exception of a single substitution, D311G, in the Sofjin (Vector) strain.

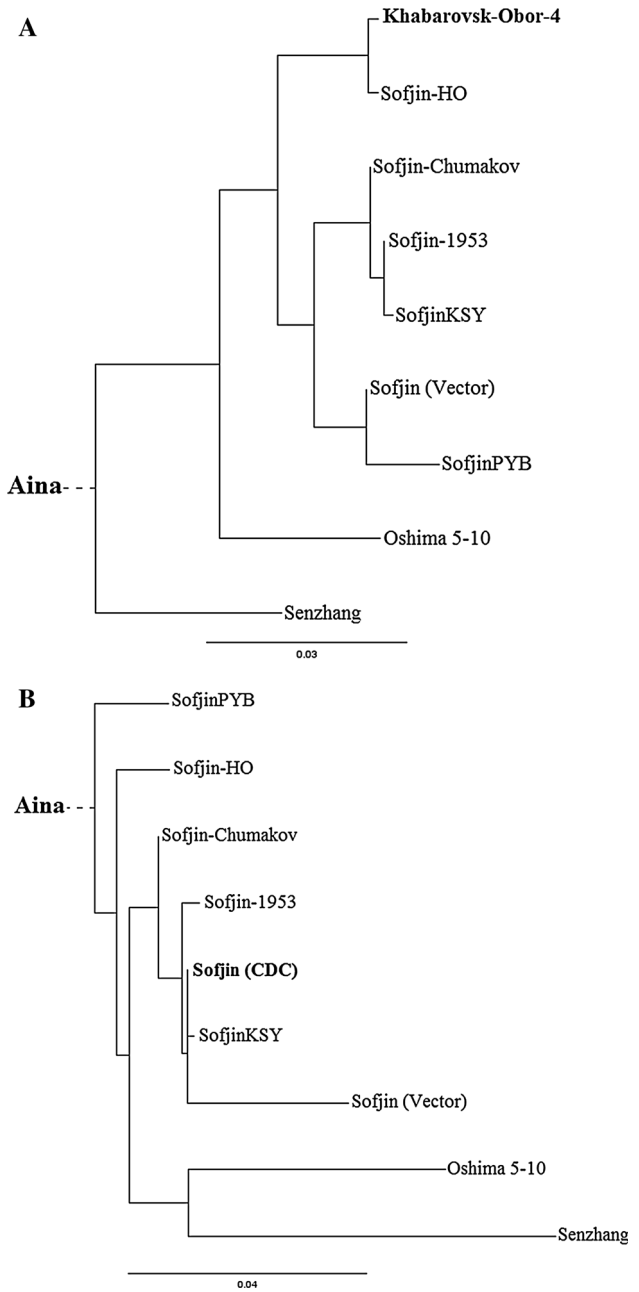


**Fig. 3** ML phylogenetic tree of the strains of tick-borne encephalitis virus. **A** Analysis of the E gene. **B** Analysis of fragments of the NS5 gene

Four substitutions in Sofjin-PYB probably can be explained by reading errors (Table 4b).

## Discussion

The primary goal of this study was the comparative analysis of the biological, molecular and genetic characteristics of 10 TBEV strains isolated before 1960 that had been stored lyophilized for up to 65 years. First, it is important to note that all TBEV strains can remain viable for a prolonged period upon storage under such conditions. The strains could be recovered by the first passage in 2-day-old mice. The biological properties of these strains described in this study were similar to those we have studied before [3]. Their specific feature was that all of them belong to a single Far Eastern TBEV subtype comprising three clusters of Sofjin-, Oshima- and Senzhang-like strains [4].

The Sofjin-like cluster includes the strains studied here that were isolated from clinical cases of TBE as well as strains isolated from ticks. The Oshima-like cluster includes strains with various biological characteristics. The Oshima 5–10 strain and similar ones were isolated in Japan from various sources (dog, tick, and rodent) in a natural focus in Hokkaido [10–12], where a case of TBE was reported in 1993, which ended in the patient's recovery. Though Oshima strains have been described by some authors [13, 14] to be typical of the Far Eastern TBEV subtype, their epidemic significance in Hokkaido (Japan) is low, and the incidence of tick-borne disease has not been reported so far. We have shown previously that the cluster of Oshima-like strains mainly consists of strains that are not pathogenic for humans, but a few strains isolated in the 1990s in the Far East from patients with manifest forms of infection are also located in this cluster [3]. Strains analyzed in this study (Primorye-1284, isolated from the blood of a patient with a febrile form of the disease, and Golubnichiy, isolated from the brain of a deceased patient with a focal form of the disease) also belong to this cluster. The results of the study allow us to conclude once again that, despite the prevalence of strains with low human pathogenicity, this group may include strains that are capable of causing manifest forms of the infection. This shows that there is no absolute correlation between the severity of infection and the molecular structure of the viral genome.

The Primorye-512 strain, isolated from mouse brain (*Apodemus peninsulae)*, belongs to the same branch of the phylogenetic tree as the Senzhang strain. The latter was isolated from the brain of a fatal case in 1953 [15]. The results of the studies convinced us that Northern Chinese strains form an independent cluster of the Far Eastern TBEV subtype [15]. This also includes some regional strains we have already studied [4, 16].

The results of analysis of the TBEV population over an 80-year period indicate that the evolution of this virus took place long before the split of the Far Eastern virus population into Sofjin-, Oshima- and Senzhang-like strains. The existence of strains belonging to different clusters of the Far Eastern TBEV population was not considered during the verification of TBE infection cases, which led to the under-diagnosis of the disease incidence and overestimate of mortality rates in the Far East [17].

**Table 4** Amino acids substitutions in fragments of E protein (A) and NS5 protein (B)

| | 55 | 59 | 141 | 151 | 159 | 248 | 294 | 346 | 351 | 359 | 500 | 501 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (a) | | | | | | | | | | | | |
| Sofjin-1953 | N | A | A | A | K | H | M | I | I | N | V | V |
| Sofjin-Chumakov | D | * | * | * | * | * | * | * | * | * | * | * |
| SofjinKSY | N | * | V | * | * | * | * | * | * | * | * | * |
| Khabarovsk-Obor-4 | D | * | V | * | * | Q | * | * | T | * | * | * |
| Sofjin-HO | D | * | V | * | * | Q | * | * | T | * | * | * |
| Sofjin (Vector) | D | * | * | * | * | Q | * | * | T | * | * | * |
| SofjinPYB | D | S | * | * | R | Q | * | M | T | * | * | * |
| Senzhang | D | * | * | P | * | Q | * | * | T | T | * | * |
| Oshima 5-10 | D | * | * | * | * | Q | V | * | T | * | M | A |

| | 58 | 130 | 173 | 181 | 189 | 216 | 231 | 235 | 264 | 287 | 311 | 317 | 325 | 330 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (b) | | | | | | | | | | | | | | |
| Sofjin-1953 | H | I | S | L | D | G | I | E | A | W | D | L | V | T |
| Sofjin-Chumakov | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| SofjinKSY | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Sofjin(CDC) | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Sofjin-HO | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Sofjin (Vector) | * | * | * | * | * | * | * | * | * | * | G | * | * | * |
| SofjinPYB | * | * | * | * | * | * | * | D | T | R | * | * | * | A |
| Senzhang | Y | V | * | * | N | R | * | * | * | * | * | * | I | * |
| Oshima 5-10 | Y | * | T | F | * | R | V | * | * | * | * | F | * | * |

The history of TBE is relevant to the studies of the Sofjin strain, which was first isolated from the brain of a patient who died from TBE in Primorsky Krai [5]. A TBEV strain that bears the name Sofjin can be found in almost all Russian and many foreign virology laboratories. There have been numerous articles claiming the true characteristics of Sofjin strain used in various virology laboratories [1, 9, 11, 13, 18]. In our studies, the Sofjin strain submitted to the Scientific Research Institute of Epidemiology and Microbiology (Vladivostok) by E. N. Levkovich in 1953 has shown high pathogenic potential. Despite the lyophilization and prolonged storage period, its neurovirulent and neuroinvasive properties have reached the highest values. This is likely to be due to numerous passages that took place before 1953, which led to the elimination of minor variations. For example, in 1938 alone, E. N. Levkovich carried out about 50 passages in white mice to ensure stable performance of Sofjin strain [1]. It appears that serial passages of isolates accompanied by selection of highly virulent clones ensured the subsequent stability of the strain's virulence properties. Despite the lyophilization and prolonged storage period, the degree of neurovirulence and neuroinvasiveness of the Sofjin-1953 strain has not changed. Similar conclusions have been made by other authors in the course of studying the biological properties of the 205 strain used for making the "Encevir" vaccine [19].

Even more convincing results have been obtained through whole-genome sequencing of the Sofjin-1953 strain, with its molecular and genetic characteristics having turned out to be similar to those of the Sofjin–Chumakov and SofjinKSY strains. Other Sofjin-like strains (Sofjin-HO, SofjinPYB and Sofjin (Vector)) differ in their molecular and genetic structure from their ancestor, i.e., the Sofjin strain isolated by L. A. Zilber, and occupy other positions on the phylogenetic tree. Therefore they cannot be classified as belonging to the group of Sofjin prototype strains. Furthermore, based on E protein sequence, the Sofjin-HO strain has turned out to be identical to Khabarovsk-Obor-4, which was isolated by the members of the northern team in the Khabarovsk region in 1937 [1].

Our results indicate that the Sofjin-1953, Sofjin–Chumakov and SofjinKSY strains, which have similar molecular and genetic structures, should be considered the standard prototypes of the Far Eastern TBEV subtype. It appears that SofjinCDC can also be assigned to this strain group based on the NS5 gene, which is the only portion of its genome whose sequence is known. The Sofjin-1953, Sofjin–Chumakov, SofjinKSY, and, probably, SofjinCDC strains can be used as reference strains, not only for

comparative analysis of the TBEV population but also for the development of reliable tick-borne encephalitis vaccines.

**Compliance with ethical standards**

**Conflict of interest** Author Galina N. Leonova declares that she has no conflict of interest. Author Sergei I. Belikov declares that he has no conflict of interest. Author Ilya G. Kondratov declares that he has no conflict of interest.

**Ethical approval** Animal experiments were performed according to the sanitary and epidemiological rules in the Russian Federation SR 1.3.3118-13 of 28.11.2013. Experiments were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, Strasbourg, 18.III.1986. url: http://conventions.coe.int/Treaty/en/Treaties/Html/123.htm.

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