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Comprehensive assessment of the genetics and virulence of tick-borne encephalitis virus strains isolated from patients with inapparent and clinical forms of the infection in the Russian Far East

Galina N. Leonova [a,*], Sergei I. Belikov [b], Ilya G. Kondratov [b], Ikuo Takashima [c]

[a] Institute of Epidemiology and Microbiology, Siberian Branch of Russian Academy of Medical Sciences, Vladivostok, Russian Federation
[b] Limnological Institute, Siberian Branch of the Russian Academy of Sciences, Irkutsk, Russian Federation
[c] Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

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ABSTRACT

We analyzed the genetics and virulence of 35 strains of TBEV isolated from patients with different forms of the infection living in the southern Far East region of Russia. The results of moleculargenetics studies of the TBEV strains showed that most of the strains that cause inapparent infections form a single cluster (I) with the Oshima 5–10 strain from Japan on the phylogenetic tree. A comparison of the amino acid sequences of the viral polyproteins of the studied strains identified 17 amino acid residues distributed unevenly across the polyprotein that distinctly differed between the clusters of inapparent and virulent strains. We detected additional substitutions in the NS1 and NS5 proteins. These substitutions might influence the pathogenic potential of the strains. Using a model of inbred mice of different ages, we examined the virulence of these strains and showed the different pathogenic potentials of strains belonging to different clusters.

Introduction

Tick-borne encephalitis (TBE) in the Russian Far East is of particular interest to scientists, primarily because it was in this region that the disease was first discovered, its etiology determined, its epidemiology described, and the basic functioning of its natural focus was studied (Zilber, 1939). Since its discovery, the idea has persisted that the classical TBE form of the Far Eastern subtype is characterized by an extremely severe infection with a high rate of unfavorable outcomes. As a rule, 67% of all TBE cases result in severe focal forms of the disease, including those resulting in fatal outcomes (32%), in patients who have not been vaccinated against TBE (Leonova, 2009). At the same time, data from the past two decades show that along with the severe focal forms of TBE, mild febrile forms of the infection have been increasingly reported (up to 50% of cases) in the Far East (Voronok et al., 2009). The clinical manifestations caused by the TBE virus (TBEV) range from inapparent infections and fevers with complete recovery of patients to debilitating or fatal encephalitis. The proportion of fatal human infections varies widely in different regions and in different years. The factors that determine disease severity are poorly defined, but correlations between viral subtypes and disease severity have been described. TBEV strains are currently divided into three closely related subtypes, Western-European (WE), Siberian (SIB) and Far Eastern (FE) (Heinz et al., 2000). FE TBEV is recognized as the most virulent subtype, with a 20–40% case fatality rate (Leonova, 2009), while the SIB subtype is considered less virulent (7–8% case fatality rate) (Gritsun et al., 2003), and the WE strains are the least virulent, with case fatality rates lower than 2% (Dumpis et al., 1999). However, a range of clinical manifestations from asymptomatic to encephalitic is observed for all of the TBEV subtypes (Gritsun et al., 2003), and the underlying basis of this range has not yet been adequately explained (Gritsun et al., 2003).

The utilization of molecular genetics methods to study TBEV has considerably increased the understanding of the structural and functional characteristics of the causative agent. In 1989, A.G. Pletnev and co-authors first deciphered the complete nucleotide and amino acid sequences of the TBEV genome (Pletnev et al., 1990) from the Sofjin strain, which originated in the Far East and was isolated in 1937 from the brain of a dead patient. Thus far, only a few TBEV strains have undergone complete genome sequencing. Most sequencing studies have been performed on the structure and functions of the E protein, which is the most important component of the outer surface of the virion envelope (Rey et al., 1995). The E protein not only determines the tropism of the virus, but in many respects, it also plays a key role in its virulence and in the induction of humoral and cellular immunity.

* Corresponding author. Fax: +7 4232 441147.
E-mail address: galinaleon41@gmail.com (G.N. Leonova).

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Other genes are also actively involved in the pathogenic potential of the TBEV strains, but their functions are not well studied.

We collected TBEV strains isolated from patients with inapparent and clinical forms of the infection, i.e., those strains with a known degree of pathogenicity in humans. We aimed to understand why the TBEV population circulating in the southern part of the Russian Far East in some cases resulted in patients with severe disease with a fatal outcome, while in other cases, patients had no clinical signs of infection, regardless of the TBEV strain. We therefore isolated TBEV strains from patients with different degrees of clinical symptoms.

The aim of this work was to sequence the complete genomes of TBEV strains that caused asymptomatic, febrile, and focal forms of infection in people from the Far East and then characterize the virulence of these strains.

Results

Genome sequence analysis

We identified the nucleotide sequences for the complete genomes of 35 TBEV strains. A comparison of the identified sequences and construction of phylogenetic trees showed that all strains, regardless of the disease severity, are related to strains of the Far Eastern subtype (Fig. 1). In addition, most of the strains that caused inapparent infections formed a separate cluster, I, in the phylogenetic tree together with the previously reported Oshima 5–10 strain from Japan. The remaining strains, which primarily caused clinical forms of TBE, formed two clusters (II and III). Cluster II included the prototype strain of the Far Eastern subtype, the Sofjin strain, and cluster III included the Senzhang strain from North China. As exceptions, two strains, Primorye-86 and Primorye-90, which caused severe focal forms of TBE, were located in cluster I, and three strains from inapparent forms of TBE, Primorye-739, Primorye-52, and Primorye-196, were grouped into clusters II, III and III, respectively. The Shkotovo-94 strain, which caused a febrile form of TBE, was intermediate between clusters I and II. The other strains from the febrile form, Kiparis-94, Primorye-82, and Primorye-94, were grouped into clusters I and II, respectively.

An analysis of the amino acid sequences of the polyprotein indicated that the locations of the amino acid substitutions were largely random, except for 17 positions in the polyprotein that were different between the inapparent (cluster I) and human pathogenic (clusters II and III) TBEV strains and conserved within each group. Table 1 shows the most common amino acid substitutions in the two groups of strains. An analysis of specific mutations indicated that most substitutions were synonymous; only 4 of the 17 amino acid substitutions changed the amino acid hydrophobicity. These four substitutions most likely have a crucial effect on the pathogenicity of the strains. Amino acid 111 was observed in a signal sequence of the core protein and was degenerate in the pathogenic strains, appearing as Leu, Met, or Val. At the same time, the inapparent strains lacked this amino acid residue. Residue 111 is located at the C-terminus of the protein in the signal sequence and allows the host cell signalase to recognize the protein by cleaving the N-terminus of the prM protein from the polyprotein. Based on the viral mechanisms of penetration and propagation in the host cells, the combination of the deletion at position 111 of the core protein and the replacement of a hydrophilic amino acid by a hydrophobic amino acid (S45F) in the viral NS3 protease may be the key substitutions.

Furthermore, we detected a S141G substitution in the NS1 protein, but it is unclear as to whether this substitution could affect pathogenicity. In the NS5 protein constituting the RNA

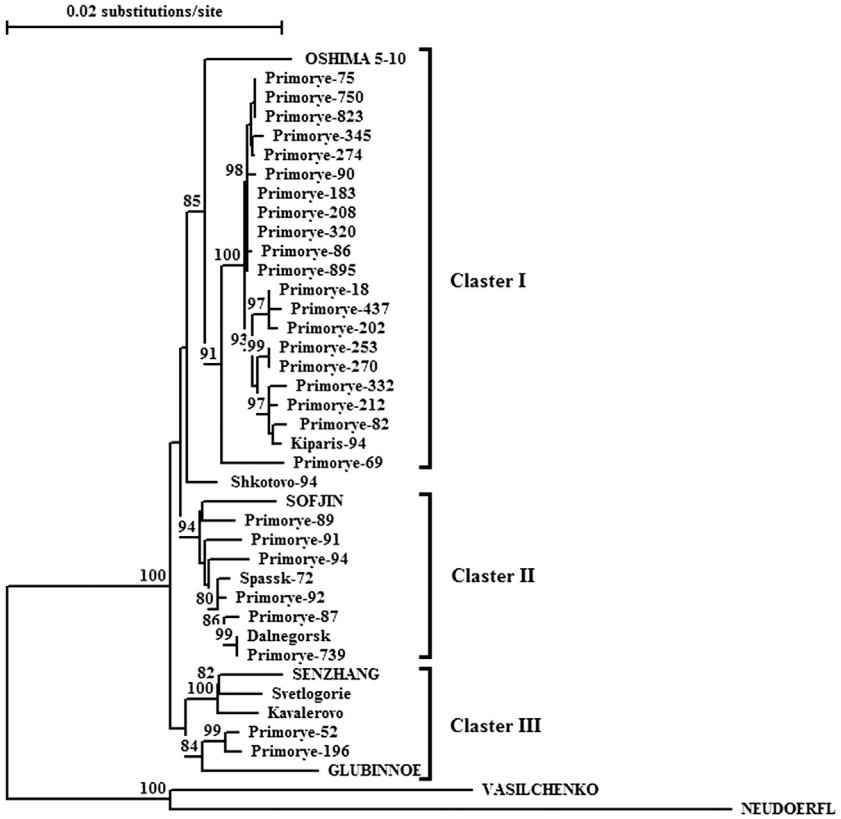

Fig. 1. Phylogenetic tree based on complete genome sequencing of the Far Eastern TBEV strains which caused inapparent, febrile, and severe focal forms of TBE. Cluster I—Oshima-like TBEV strains, cluster II—Sofijn-like TBEV strains, and cluster III—Senzhang-like TBEV strains.

Table 1
Substitutions of amino-acid residues in TBEV proteins characteristic of the strains with different virulence in humans. Single substitutions which do not coincide with the typical group-specific substitutions are marked in gray.

	Capsid				prM	E	NS1	NS2B	NS3		NS4B			NS5			
	32	69	100	111	151	463	141	108	16	45	95	179	213	634	677	692	724
SOFJIN	**Q**	**K**	**D**	**L**	**A**	**V**	**S**	**F**	**R**	**S**	**M**	**V**	**A**	**S**	**G**	**I**	**A**
Primorye-89	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**R**	.	.
Primorye-91	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Primorye-92	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Spassk-72	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Primorye-94	.	.	.	.	.	.	.	.	.	.	.	**A**	.	.	.	.	.
Primorye-87	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Dalnegorsk	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Primorye-739	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GLUBINNOE	.	.	.	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.
Primorye-196	.	.	.	**M**	.	.	.	.	.	.	.	.	.	.	.	.	.
Primorye-52	.	.	.	**M**	.	.		.	.	.	.	.	.	.	.	.	.
SENZHANG	.	.	.	**V**	.	.	.	.	.	.	.	.	.	.	.	.	.
Kavalerovo	.	.	.	**V**	.	.	.	.	.	.	.	.	.	.	.	.	.
Svetlogorie	.	.	.	**V**	.	.	.	.	.	.	.	.	.	.	.	.	.
Shkotovo-94	.	.	.	.	.	.	.	.	**K**	**F**	.	.	.	.	.	.	.
OSHIMA 5-10	**R**	.	.	**V**	**V**	**A**	.	.	.	**F**	.	.	.	**T**	.	**V**	.
Primorye-69	**R**	**R**	.	**V**	**V**	**A**	**G**	.	**K**	**F**	.	.	**V**	**T**	**K**	**V**	**S**
Primorye-437	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-202	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-18	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-895	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-183	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-320	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-208	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-86	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-274	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-750	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-75	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-828	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-823	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-90	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-345	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-270	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-253	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-212	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-332	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**
Kiparis-94	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	.	**A**	**V**	**T**	**K**	**V**	**S**
Primorye-82	**R**	**R**	**N**	Del	**V**	**A**	**G**	**V**	**K**	**F**	**V**	**A**	**V**	**T**	**K**	**V**	**S**

polymerase domain, we identified four substitutions, three of which were conserved (S634T, R677K, and I693V) and one of which (A724S) changed the hydrophobicity of the amino acid residue.

We also identified one synonymous amino acid substitution in each membrane protein of the virus strain groups with different pathogenicities. Moreover, each substitution is located at a transmembrane site. Such substitutions should not affect the conformation of either the prM or E proteins, and thus, they cannot affect the pathogenicity of the TBEV strains.

Virulence analysis in a mouse model

Subsequently, we focused on studying the virulence properties of the isolated and sequenced TBEV strains that had caused the different clinical presentations (focal, febrile, and inapparent) in humans.

All TBEV strains were isolated following the injection of two-day-old inbred albino mice. On this basis, we concluded that this animal model is highly susceptible to the isolation of the strains causing both inapparent and clinical forms of the infection in humans. To understand the features of the strains capable of causing different degrees of pathology in humans, we comprehensively studied their virulence in albino mice of different age groups as an important biological characteristic.

To study the virulence of the TBEV strains, we used albino mice aged 1.5–2 months (weight 8–10 g) because all of the two-day-old mice inoculated with TBEV strains from different TBE forms died on days 5–7 and we failed to identify differences in the development of the infection in these mice. We determined the virus titer of the strains in brain tissue after the intracerebral and subcutaneous injection of albino mice. Table 2 shows that after the intracerebral injection of animals, the virus titer in all groups was roughly the same and averaged $10^{9.4\pm0.33}$ in Group 1, $10^{9.8\pm0.35}$ in Group 2, and $10^{9.2\pm0.23}$ LD_{50}/ml in Group 3. By contrast, after the subcutaneous injection, the mean titers were $10^{8.2\pm0.45}$, $10^{8.1\pm0.36}$, and $10^{7.0\pm0.25}$ LD_{50}/ml, respectively. The invasiveness index averaged 1.16 ± 0.28, 1.8 ± 0.16, and 2.2 ± 0.22, respectively, indicating the difference in the neuroinvasive properties of the strains in these groups. In Groups 1 and 2, the neuroinvasiveness index values were typically ≤2.0, indicating high neuroinvasive activity of these strains. In Group 3, the invasiveness index ranged from 0.7 to 4.5, suggesting the presence of strain variations with high, moderate, and low neuroinvasiveness.

We studied the virulence of the strains in the adult albino mouse model (weight 18–20 g) by intracerebral and subcutaneous injection with different doses (10 and 1000 LD_{50}) or the same dose (100 LD_{50}) of the virus (Table 3). When mice were injected intracerebrally, the strains of all groups showed high virulence. Following the subcutaneous injection of the virus, Group 1 strains showed the highest virulence, with an average survival of

Table 2
Characteristics of the Far Eastern TBEV subtype strains that caused different clinical forms of the infection in humans.

№	Strain	Passage	Region of isolation	Year of isolation	Virus titer in white mice (lg LD_{50}/ml)		Index of invasiveness (I.I.)
					i.c.	s.c.	
	Strains caused severe focal form of TBE (group 1)						
1	Spassk-72	6	Spassk	1972	8.0	6.3	1.7
2	Dal´negorsk	8	Dalnegorsky	1973	9.8	9.5	0.3
3	Kavalerovo	V	Kavalerovsky	1985	9.1	8.2	0.9
4	Primorye-86	IX	Kirovsky	1986	10.0	9.5	0.5
5	Primorye-87	V	Kavalerovsky	1987	8.0	6.7	1.3
6	Primorye-89	VII	Arseniev (town)	1987	9.8	7.9	1.9
7	Primorye-90	VII	Arseniev (town)	1990	10.0	9.7	0.3
8	Primorye-92	IV	Vladivostok (city)	1992	10.5	8.1	2.4
	$M \pm m$				9.4 ± 0.33	8.2 ± 0.45	1.16 ± 0.28
	Strains caused febrile form of TBE (group 2)						
1	Primorye-82	IX	Vladivostok (suburbs)	1982	10.1	8.7	1.4
2	Shkotovo-94	V	Shkotovo	1994	9.1	7.5	1.6
3	Primorye-94	VI	Nadezhdinsky	1994	9.5	7.4	2.1
4	Kiparis-94	III	Nadezhdinsky,Kiparisovo	1994	10.7	8.7	2.0
	M ± m				9.8 ± 0.35	8.1 ± 0.36	1.8 ± 0.16
	Strains caused inapparent form of TBE (group 3)						
1	Primorye-183	IV	Nadezhdinsk	1991	9.3	7.5	1.8
2	Primorye-212	II	Vladivostok (suburbs)	1991	10.5	6.9	3.6
3	Primorye-253	II	Nadezhdinsky, Solovey Kluch	1991	8.3	6.7	1.6
4	Primorye-270	III	Nadezhdinsky, Mirny	1991	8.3	6.9	1.4
5	Primorye-332	V	Nadezhdinsky	1991	8.5	6.7	1.8
6	Primorye-739	IV	within the city of Vladivostok	1992	8.8	6.2	2.6
7	Primorye-18	V	Vladivostok (city)	1997	11.0	7.7	3.3
8	Primorye-202	VI	Nadezhdinsky, Solovey Kluch	1997	11.0	7.3	3.7
9	Primorye-750	III	Nadezhdinsky, Kiparisovo	1998	9.0	6.4	2.6
10	Primorye-828	IV	Chernigovka (village)	1998	9.3	7.3	2.0
11	Primorye-52	V	Shkotovsky, Anisimovka	1999	9.1	7.9	1.2
12	Primorye-75	II	Nadezhdinsk	1999	10.5	7.7	2.8
13	Primorye-274	III	Nadezhdinsk	1999	9.2	8.0	1.2
14	Primorye-345	V	Vladivostok (suburbs)	1999	9.0	6.7	2.3
15	Primorye-320	II	Shkotovo	1999	10.3	8.9	1.4
16	Primorye-437	VIII	Area of bay Lazurnaya	1999	7.7	5.5	2.0
17	Primorye-69	VI	Ussuriysk	2000	7.7	7.0	0.7
18	Primorye-196	VII	Area of bay Lazurnaya	2000	8.2	3.7	4.5
19	Primorye-823	II	Krasnoarmeysky	2000	9.5	7.7	1.8
	$M \pm m$				9.2 ± 0.23	7.0 ± 0.25	2.2 ± 0.22

11.2 ± 1.2 days following an injection of 1000 LD_{50} and 13.6 ± 1.7 days following an injection of 100 LD_{50}. Group 2 strains showed the lowest virulence, with average survival times of 14.0 ± 2.5 and 22.5 ± 2.3 days, respectively. In Group 3, strains from the inapparent TBE form were divided into two subgroups, highly invasive strains with average survival times of 9.6 ± 0.5 and 14.6 ± 0.8 days and minimally invasive strains with average survival times of 17.1 ± 1.9 and 22.4 ± 1.6 days, respectively. Fig. 2 shows the sequential mortality of strains Spassk-72, Shkotovo-94, and Primorye-437, which represent the different (1, 2, 3) groups; the data demonstrated that Spassk-72 had a higher neurovirulence and neuroinvasiveness than Shkotovo-94 and Primorye-437. The results of these experiments indicate that the degree of neuroinvasiveness of the TBEV strains is best identified in adult mice injected subcutaneously with infectious inocula.

Viral replication and pathogenesis of TBEV in a mouse model

We studied the pathogenesis of TBEV in a mouse model injected with different TBEV strains: the Spassk-72 strain isolated from the brain of a dead patient with the severe focal form of TBE, the Shkotovo-94 strain isolated from the blood of a patient with the febrile form of TBE, and the Primorye-437 (P-437) strain isolated from the blood of a patient with the inapparent form of TBE. As shown in Fig. 2, the virulence of these strains varied with the intracerebral (10 LD_{50}) and subcutaneous ($10^{3.0}$ LD_{50}) injection of the animals, i.e., the neuroinvasiveness of these strains was different. Observation of the dynamics of viral replication in the organs (Fig. 3A,B,C in the blood, in the spleen, and in the brain respectively) of albino mice allowed us to obtain additional data on the virulence of the studied strains. Fig. 3 shows that the infection developed differently with the same infecting dose (10^3 $TCID_{50}$) for the three strains. TBEV strains that had caused clinical forms of the disease in humans appeared in the blood of albino mice on day 3; the highest value was registered for Spassk-72 (10^4 $TCID_{50}$). P-437 appeared in the blood of the experimental animals on day 5 with a low titer (10 $TCID_{50}$). The viremic peak for the strains in cluster II (Spassk-72 and Shkotovo-94) occurred on day 5, while it occurred on day 11 for the strain in cluster I (P-437). In the spleen, the viremic peaks for Spassk-72 and Shkotovo-94 also occurred on day 5, with higher titers compared to the blood. For P-437, the peak was again observed on day 11. The viremic peak in the brain (10^6 $TCID_{50}$) for Spassk-72 was observed from days 9–11 (the time of death of the animals). For Shkotovo-94, the maximum value (4 log TCD_{50}) was observed on day 7. For P-437, the virus appeared in the brain of the injected albino mice on day 7 (10 $TCID_{50}$), and the virus titer then gradually increased, reaching its maximum peaks on day 11 (10^4 $TCID_{50}$) and day 14 (10^6 $TCID_{50}$).

Changes in the weight (Fig. 4A) and body temperature (Fig. 4B) of the albino mice injected with the same dose (10^3 $TCID_{50}$) of the TBEV strains were used to indicate the severity of the infection. Fig. 4A shows that Spassk-72, which was isolated from the brain of a dead patient, caused rapid weight loss in mice followed by death. Animals injected with Shkotovo-94 began to lose weight rapidly,

Table 3
Survival rate and average life expectancy of mice injected with the regional TBEV strains of the Far Eastern subtype.

No.	Strains	Survival rate (%)				Average life expectancy of mice (days)			
		i.c. 10 LD_{50}	s.c. 1000LD_{50}	i.c. 100 LD_{50}	s.c. 100 LD_{50}	i.c. 10 LD_{50}	s.c. 1000LD_{50}	i.c. 100LD_{50}	s.c. 100LD_{50}
	Strains caused severe focal form of TBE (group 1)								
1	Spassk-72	0	0	0	0	6.6	11.5	5.6	8.9
2	Dal´negorsk	0	0	0	30	6.4	7.1	5.0	14.7
3	Kavalerovo	0	0	0	10	5.2	8.1	6.5	13.1
4	Primorye-86	0	0	30	80	15.3	16.0	14.6	24.9
5	Primorye-87	0	30	0	10	6.7	17.3	6.6	14.3
6	Primorye-90	0	0	0	0	7.9	10.3	5.8	11.1
7	Primorye-91	0	0	0	0	6.7	9.2	5.3	9.3
8	Primorye-92	20	0	0	10	10.4	9.8	6.9	12.3
	$M \pm m$	2.5 ± 2.5	3.75 ± 3.7	3.75 ± 3.75	17.5 ± 9.6	8.15 ± 1.2	11.2 ± 1.2	7.0 ± 1.1	13.6 ± 1.7
	Strains caused febrile form of TBE (group 2)								
1	Primorye-82	0	0	0	60	8.7	7.7	8.1	23.6
2	Shkotovo-94	40	40	50	80	18.3	19.6	17.2	24.4
3	Primorye-94	80	40	60	90	25.2	16.0	22.3	26.3
4	Kiparis-94	0	30	0	10	7.0	12.8	6.4	15.7
	$M \pm m$	30 ± 19.1	27.5 ± 7.5	27.5 ± 16.0	60 ± 17.8	14.8 ± 4.2	14.0 ± 2.5	13.5 ± 3.8	22.5 ± 2.3
	Strains caused inapparent form of TBE (group 3)								
A. Highly invasivness									
1	Primorye-183	0	10	0	10	6.5	10.9	6.4	12.2
2	Primorye-332	0	10	0	20	7.7	11.1	5.9	13.3
3	Primorye-739	0	0	0	10	5.4	7.7	6.6	12.4
4	Primorye-52	0	0	0	30	7.2	9.1	7.3	16.0
5	Primorye-75	10	0	10	10	11.1	9.4	10.3	13.4
6	Primorye-274	0	0	0	20	5.8	9.1	8.6	14.4
7	Primorye-320	0	0	0	10	10.3	11.8	7.0	13.3
8	Primorye-202	0	0	0	40	7.6	8.4	7.0	18.7
9	Primorye-828	0	0	0	40	7.0	8.6	7.0	17.7
	$M \pm m$	1.11 ± 1.1	2.2 ± 0.5	1.11 ± 1.1	21.1 ± 4.2	7.6 ± 0.6	9.6 ± 0.5	7.3 ± 0.4	14.6 ± 0.8
B. Low invasivness									
1	Primorye-270	40	90	0	100	17.0	26.2	8.3	28.0
2	Primorye-18	0	10	10	90	15.9	16.5	10.5	26.3
3	Primorye-345	70	0	10	40	21.3	11.9	10.0	18.5
4	Primorye-437	70	60	0	60	22.4	25.1	8.0	22.1
5	Primorye-69	0	0	50	100	6.4	13.6	19.2	28.0
6	Primorye-196	10	60	0	90	8.1	21.1	4.6	26.2
7	Primorye-823	20	0	0	40	10.1	8.9	6.7	17.1
8	Primorye-212	0	10	0	30	6.5	16.3	6.4	18.0
9	Primorye-253	0	20	0	30	8.4	14.6	6.1	17.6
	$M \pm m$	23.3 ± 9.9	27.8 ± 11.1	7.8 ± 5.4	64.4 ± 10.1	12.9 ± 2.1	17.1 ± 1.9	8.9 ± 1.4	22.4 ± 1.6

with a decrease in body temperature starting on day 9 before the end of the experiment. Animals injected with the P-437 strain (isolated from an inapparent infection) gained weight through day 8 and then gradually began to lose weight.

Discussion

The long-standing notion that the Far Eastern subtype TBEV strains cause only severe forms of TBE is gradually dissipating. Over the past 20 years, we have conducted research aimed at isolating and characterizing TBEVs causing clinical courses of varying severity, from inapparent to severe focal forms of TBE (Leonova et al., 2007). We have shown that the Far Eastern viruses have heterogeneous virulence in humans; the bulk of these infections (as high as 70%) are caused by non-neuroinvasive strains, which are rapidly and completely eliminated from the human body following the tick bite. Additionally, neuroinvasive strains causing lethal infections in albino mice comprise a relatively small proportion of the virus population (as much as 30%).

In this study, we obtained the molecular structure and determined the degree of virulence of these TBEV strains, allowing us to identify characteristics related to the pathogenic potential of the strains of the Far Eastern TBEV subtype, which are capable of causing both inapparent and clinical forms of TBE.

The molecular genetics analyses of 35 TBEV strains causing different levels of disease severity demonstrated that the bulk of the strains that cause inapparent infections formed a separate cluster (I) in the phylogenetic tree near the previously reported Oshima 5–10 strain from Japan (Takashima et al., 1997). Two other clusters with the prototype strains Sofjin (II), which was isolated in 1937 in the Russian Far East, and Senzhang (III), which was isolated in 1953 in China, primarily include human pathogenic strains capable of causing clinical forms of the disease.

Recent studies on the biology of viruses have considered virulence to be a phenotype of the polygenic inheritance of pathogenicity (Heinz, 2003). Hence, if pathogenicity is a constant species characteristic, then virulence is its individual strain variable (McMinn, 1997). In current studies included not only obtaining the complete genomic characteristics of the TBEV strains but also determining their virulence to identify correlations between these major characteristics and their influence on the course of infection in patients with TBE.

We have primarily shown that the studied TBEV strains were different in some of their biological properties (neurovirulence and neuroinvasiveness). They had rather high pathogenic potential in albino mice when injected intracerebrally, particularly in two-day-old animals and animals weighing 8–10 and 18–20 g. Therefore,

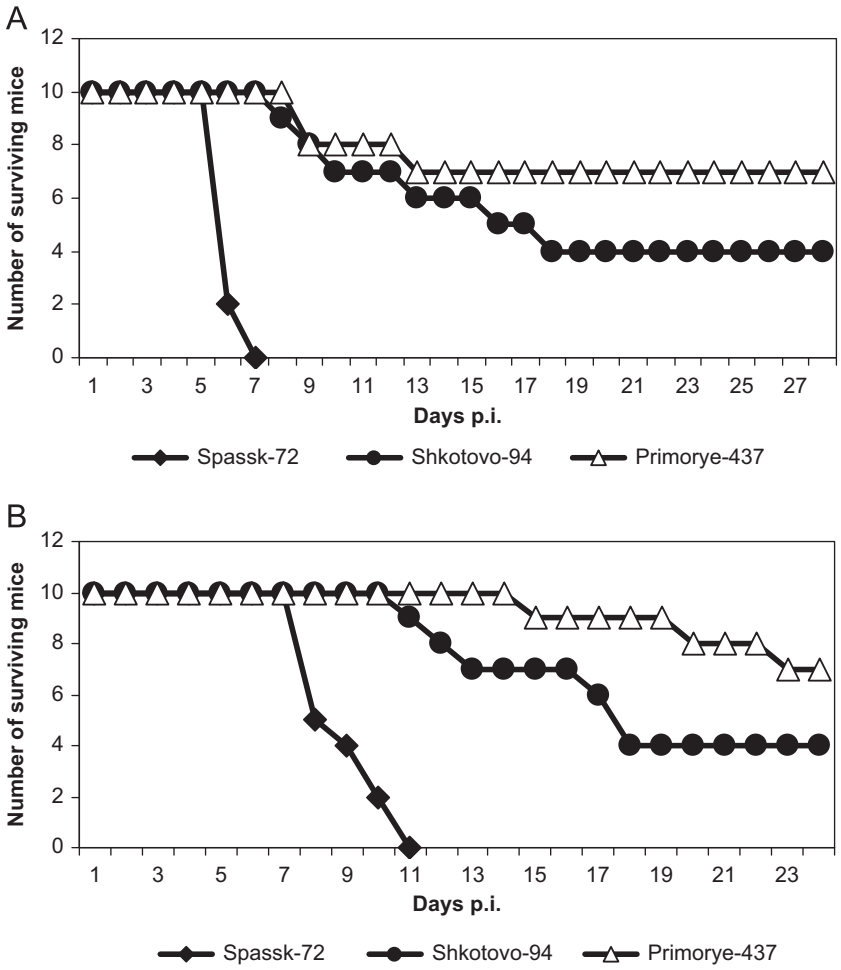

Fig. 2. Survival rate of mice injected with the strain Spassk-72 (caused severe focal form of TBE; related to the cluster II), Shkotovo-94 (caused febrile form of TBE; related to the cluster II), and Primorye-437 (caused inapparent form of TBE; related to the cluster III) A—intracerebral injection (10 LD_{50}); B—subcutaneous injection (1000 LD_{50}).

only parameters for peripheral activity allowed us to identify the differences between the studied strains. In the group containing strains causing inapparent infection, the pathogenic potential was significantly lower compared to the strains causing severe focal TBE.

In recent years, studies have attempted to identify the molecular mechanisms of viral neurovirulence and neuroinvasiveness (Karganova, 2009; Goto et al., 2002; Gritsun et al., 2003; Gritsun et al., 2003; Hayasaka et al., 1999; Hayasaka et al., 2001; Tamura et al., 2007). In fact, the specific TBEV clones studied by these authors have furthered the understanding of the basics of virulence in strains of the virus capable of causing different courses of infection.

There are three possible mechanisms to explain the brain tissue damage caused by the viruses. First, the virus may cause direct damage to neurons. Second, virus-induced inflammatory responses may result in neuronal death. Finally, a combination of both of these mechanisms may be the cause of severe infection (King et al., 2007).

Recently, D. Ruzek and co-authors (Ruzek et al., 2009; Ruzek et al., 2011) reported interesting data on the immunopathology of TBE and the breakdown of the blood-brain barrier (BBB) in experimentally injected non-inbred mice with different immunodeficiencies. The authors identified two damaging factors in normal mice with fully developed immune systems: the direct destructive effect of the virus on the cells of the central nervous system (CNS) and the immunopathological response. These factors influence the clinical outcome of the disease in humans and animals (Ruzek et al., 2009). Changes to the BBB are a characteristic feature of many CNS infections and are most likely essential for disease progression (Olsen et al., 2007). Considering the above results on TBEV accumulation in a laboratory model of albino mice (Fig. 3), we suggest that animals injected with the Spassk-72, Shkotovo-94, and P-437 strains had different courses of infection. Thus, in albino mice, we detected initial reactive changes in the BBB structures within one day of injection with the highly virulent Spassk-72 strain (the meninges and vascular plexus), and on days 5–7, we observed destructive changes in the indicator areas of the brain (the subcortical and stem structures and the cerebellum) (Somova et al., 2013). Based on the literature (Ruzek et al., 2011; Tigabu et al., 2009), in this case, we believe that the BBB broke down in mice injected with the Spassk-72 strain even at early stages of the infection. This breakdown was followed by an abrupt decrease in body weight and an increase in the virus titer in the brain accompanied by destructive changes in the cells. Although the infecting dose was low (3 log $TCID_{50}$), an acute clinical picture developed, resulting in fatal outcomes in all animals. This most likely indicates the combined mechanism of a severe course of the infection associated with the direct destruction of neurons and virus-induced inflammatory responses.

In contrast, the P-437 strain, which had caused an inapparent form of infection in humans, had a prolonged period of virus accumulation in the brains of experimentally injected albino mice (7–14 days), which showed only an insignificant reaction by the pial vessels. The individual mutations identified in the active center of the viral serine protease (NS2B/NS3 complex) most likely contributed to the delay of virus accumulation at the site of

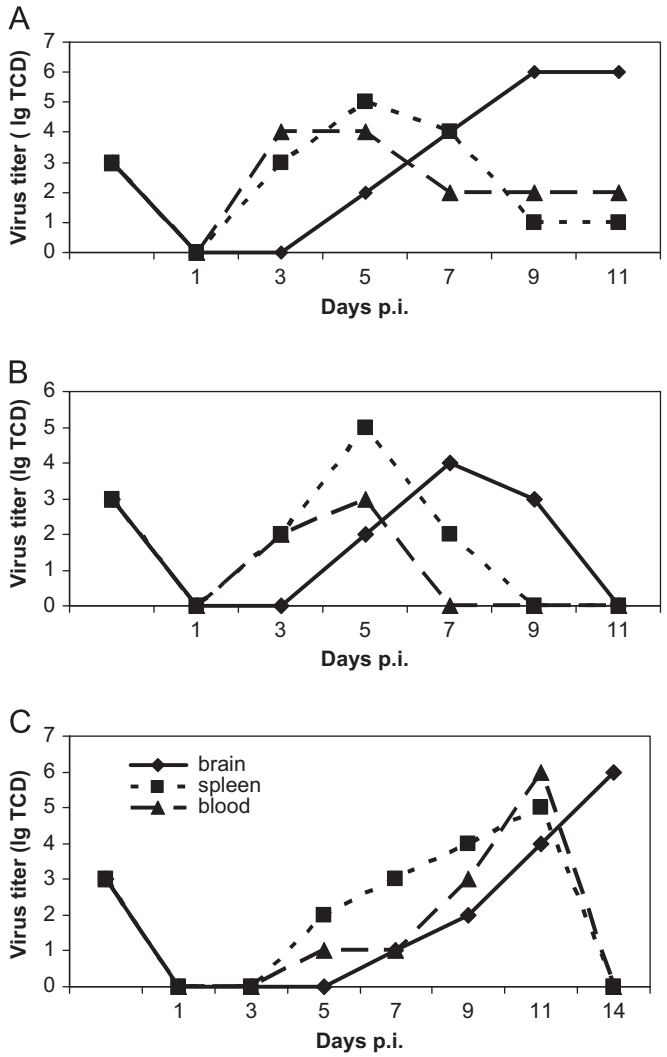

Fig. 3. Dynamics of the virus multiplication in the organs (brain, spleen, and blood) of albino mice injected with different TBEV strains: A—Spassk-72 caused focal form of TBE; B—Shkotovo-94: febrile form of TBE; C—Primorye-437: inapparent form of TBE. The horizontal axis shows the virus titer in log TCD_{50}; the vertical axis—observation days after injection.

inoculation, thus limiting virus distribution in the CNS. Other authors (Ruzek et al., 2009, 2011) have also discussed this type of damage. In later stages in mice injected with the P-437 strain, we observed widespread severe changes in neurons of a lytic type (Somova et al., 2013). Additionally, on day 8 post-injection, the animals began to lose weight, but they only began to show stable weight loss on day 14. The body temperature of the animals decreased gradually. The P-437 strain can possibly be characterized by the direct penetration of the virus into the brain without BBB breakdown. This was experimentally confirmed (Ruzek et al., 2011) when a low-virulence TBEV strain showed prolonged replication in the mouse. Consequently, the characteristic features of TBEV strains causing inapparent forms of TBE were a long-term accumulation of the virus in injected albino mice, a weak reaction of the BBB cells, and destruction of neurons in the later stage of observation.

The Shkotovo-94 strain was located in the phylogenetic tree between clusters I and II and shared 15 of 17 amino acid residues that are characteristic of the virus strains causing focal disease. Two amino acid substitutions (16Lys and 45Phe in NS3) were also characteristic of Shkotovo-94 and other inapparent virus strains. The substitutions are located in the viral protease that is part of the NS2B/NS3 protein complex. This substitution likely reduces the pathogenic potential of strains such as Shkotovo-94.

Unlike Shkotovo-94, the Primorye-82 and Kiparis-94 strains, which also cause a febrile form of the disease, do not form separate branches in the phylogenetic tree and are located in the strain group of cluster I according to the specific amino acid substitutions. They have a unique substitution of one amino acid residue, D809N, in the NS5 protein (data not shown). It is possible that such a substitution can increase the pathogenic properties of these strains; however, at present, its mechanism is unknown.

We found that the Primorye-94 strain, which causes a febrile form of TBE and localizes with the pathogenic strains of cluster II in the phylogenetic tree, contained a number of amino acid substitutions that are atypical for the groups of inapparent and pathogenic strains, including V141I in the E protein, K47R in the NS1 protein, A68V, L143I and T184I in the NS3 protein, V179A in the NS4B protein, and E878G in the NS5 protein. It can be assumed that such a combination of many amino acid substitutions may reduce the pathogenicity of the strain.

The comparison of the amino acid sequences of the viral polyproteins of the studied strains allowed the identification of 17 amino acid residues distributed unevenly across the polyprotein that differ between clusters of inapparent and virulent strains. The presence of 17 substitutions in the polyprotein is not evidence that all 17 substitutions affect the virulence of the strains. Some of these substitutions may reflect the evolutionary history of the virus and be neutral or irrelevant to the strain pathogenicity. Furthermore, some substitutions do not change the properties of the amino acid residues; therefore, they do not affect the protein conformation and thus do not affect strain pathogenicity. However, it is also possible that a combination of several irrelevant substitutions can change the conformation of the proteins and affect the pathogenicity of the strains.

The analysis indicates that a combination of the unique deletion of amino acid 111 in core protein C with the conserved substitution R16K and the significant substitution S45F, which changes the hydrophobicity of the amino acid residue in the NS3 protein (i.e., in the viral protease), are the most likely substitutions affecting strain pathogenicity. The amino acid deletion in the signal sequence of the core protein reduces the possibility of (or decelerates) the cleavage of the N-terminus of the premembrane protein. Moreover, according to the methods of homology modeling and molecular dynamics, the two substitutions in the protease, or rather in the NS2B/NS3 complex, change the conformation of the protease complex, thus decreasing its ability to bind to the substrate (Potapova et al., 2011). Mammalian cells are known (Monastyrskaya et al., 2004) to contain a DExH-like RNA helicase that displays significantly increased expression with TBEV replication. We demonstrated that substitutions in the NS2B/NS3 complex affected the replication rate of the studied strains (Table 1 and Fig. 3). A comparison of the complete nucleotide sequences of another flavivirus, the neurovirulent strain of the Japanese encephalitis virus, with the three attenuated variants identified mutations at highly conserved sites of the proteins NS2B (E63D) and NS3 (A105G) (Wallner et al., 1996).

Another significant amino acid substitution, which may change the hydrophobicity and conformation of the protein, is the S141G substitution in the NS1 protein; however, the exact role of the NS1 protein in the pathogenicity of the virus is currently unknown. In the RNA polymerase domain of the NS5 protein of inapparent strains, we found four substitutions, of which three were conserved (S634T, R677K, and I692V) and one (A724S) changed the hydrophobicity of the amino acid residue. The combination of these substitutions can affect the pathogenic potential of the strains. Determining the role of

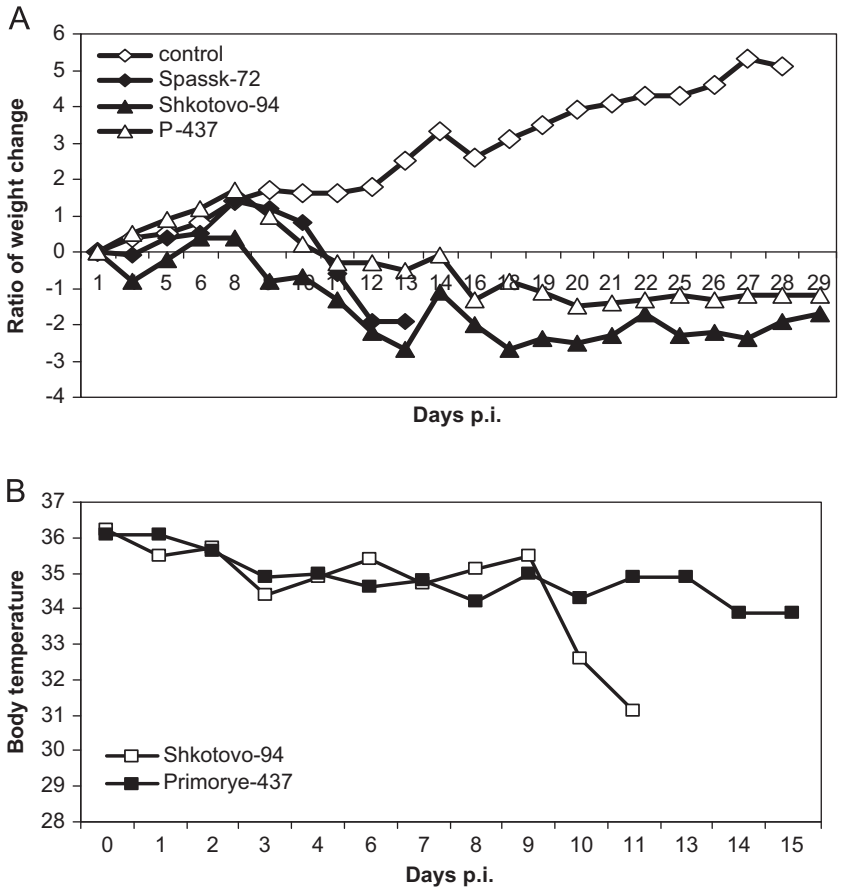

Fig. 4. Changes in weight (*A*) and body temperature (*B*) of albino mice injected with the TBEV strains which were isolated from infected patients. Note: the strains of the cluster II (Spassk-72 caused focal form of TBE and Shkotovo-94—febrile form of TBE) and the strain of the cluster I (Primorye-437 caused inapparent form of TBE).

individual amino acids in pathogenicity requires additional studies on the directed introduction of specific mutations and the assessment of their impact using reverse genetics.

Comprehensive experimental research aimed at studying the molecular characteristics and virulence of the TBEV population has shown that all TBEV strains isolated from patients, regardless of the documented form of the infection, exhibit pathogenicity in humans. However, the pathogenic potential (virulence) of the strains isolated from patients with inapparent and clinical forms of TBE differs significantly. In the blood, each TBEV strain displays genetic characteristics that can influence the occurrence of relatively favorable or dramatic events in the host.

In this report, we did not discuss the role of the mammalian immune system, which has a great influence on the fate of the causative disease agent. When inapparent TBEV strains enter the blood after a tick bite, the chances of their survival in the host are low, and as mentioned above (Leonova et al., 2007), these strains represent the bulk of the virus population. As a rule, even at the initial stage of the infection, these strains are eliminated from the host.

The strains that cause clinical forms of the infection may have a different effect in the host, which depends not only on the molecular characteristics of the causative agent but also on the state of the immune system. The latter is also known to determine different courses of infection, contributing to either the elimination of the virus followed by patient recovery or to an unfavorable outcome.

There are new opportunities for predicting the course and outcome of TBE that consider the molecular characteristics of the causative agent and the state of the host immune system at the initial stage of the infection. These opportunities should be considered when developing preventive and medical preparations.

Materials and methods

Virus strains

We examined 35 TBEV strains isolated in the southern part of the Russian Far East from patients with different forms of TBE. The strains were isolated from 2-day-old albino mice following intracerebral injection. The clinical manifestations of the infection were observed over two to three weeks. The focal strains were isolated from the brains of patients who died from TBE. The febrile strains were isolated from the blood of those patients with this form of TBE infection diagnosed in hospital infectious departments. Inapparent strains were isolated from the blood of patients on days 1–5 after sucking of antigen-positive ticks by ELISA. Since these patients had no clinical symptoms, they did not consult the doctor.

Virulence analysis

We studied the neurovirulence and neuroinvasiveness of 31 Far Eastern subtype TBEV strains causing severe focal (Group 1, $n=8$), febrile (Group 2, $n=4$), and inapparent (Group 3, $n=19$) forms of the infection (Table 1). We used the strains obtained from 3 to 9 passages in two-day-old inbred mice. Viral titers of the TBEV strains were determined through the intracerebral and subcutaneous injection of albino mice weighing 8–10 g. These indicators were calculated for 1 mL. The invasiveness index was calculated as

the difference in the values of the infectious titers between hypodermic and intracerebral injections.

We performed a comparative study of the neurovirulence of these strains by injecting mice weighing 18–20 g with different doses of TBEV. The viral dose ($\log LD_{50}$) was determined by titration of the strain in white mice using the intracerebral method of infection outlined by Hayasaka et al. (1999); mice were injected intracerebrally with 10 LD_{50} per 0.03 mL and subcutaneously with 1000 LD_{50} per 0.2 mL. In other experiments, mice were injected intracerebrally (0.03 mL) and subcutaneously (0.2 mL) with a dose of 100 LD_{50} of the virus. For each experiment, we used 10 mice, and the observation period was 28 days. The survival rate and average life expectancy of the animals served as the evaluation criteria of neurovirulence.

We studied the dynamics of the accumulation of different TBEV strains in the organs of albino mice (blood, spleen, and brain). We used the same injection dose (10^3 $TCID_{50}$) for the three strains (Spassk-72, Shkotovo-94, and Primorye-437). To determine the viral titer, continuous pig embryo kidney cells (PK cells) cultivated in medium 199 containing 7% fetal calf serum and gentamicin were used. From the brain and spleen of the experimental animals, we took 0.1 g pieces and triturated them in 1 mL mortar. We obtained 10% suspension which was centrifuged at 3–4 thousands rpm for 15 min and followed by 10-fold dilution. Overnight monolayers of PK cells grown in 24-well plates were infected with 10-fold dilutions of the experimental samples. After 1 h of contact at 37 °C, the infected cell monolayer was washed with medium 199. 199 maintenance medium containing gentamicin and 1% fetal calf serum was then added, and the plates were placed in an incubator. Observations of cytopathic effects (CPEs) were made for 5–7 days. The samples were simultaneously titrated in PK cell cultures using indicators of the virus titer by CPEs as described above.

Changes in the weight of the animals injected with the different strains were observed over four weeks, and changes in body temperature were observed for 15 days. Animals were kept in a vivarium according to the USSR Ministry of Public Health Regulation No. 1189 of 10.10.1983. Experiments were performed according to the USSR Ministry of Public Health Regulations No. 755 of 12.09.1977 and No. 701 of 27.07.1978 for the humane treatment of animals.

Genetic analysis

We determined the complete genome sequences of 35 TBEV strains isolated in the southern region of the Russian Far East from patients with different forms of TBE. Total RNA extraction was performed using the RIBO-zol-A kit (AmpliSens, Russia) according to the manufacturer's protocol. Reverse transcription and amplification of the viral RNAs were performed using a constructed set of 39 primers selected by comparison of the complete genome sequences of the TBEV strains from the GenBank database (U27495, AF069066, U39292, AB062063, AY182009, DQ862460, AB062064, and DQ989336). The reverse transcription reaction was performed using the Reverta-L-100 kit (AmpliSens, Russia) according to the manufacturer's protocol. The virus genome was sequenced using either a CEQ 8800 (Beckman Colter) or Prism 3100 (Applied Biosystems) Genetic Analyzer according to the manufacturer's protocol.

To obtain complete genome sequences of the TBEV strains, overlapping nucleotide sequences for of all of the genome fragments for each strain were aligned and pooled using the program BioEdit (http://www.mbio.ncsu.edu/BioEdit/). The nucleotide sequences were translated into amino acid sequences online at http://web.expasy.org/translate/ (Bioinformatics Resource Portal).

All nucleotide and translated amino acid sequences of the strains were deposited in the international GenBank database (http://www.ncbi.nlm.nih.gov/) under the following accession numbers: FJ402886—Dalnegorsk, FJ402885—Kavalerovo, JQ825146—Kiparis-94, GQ228395—Primorye-18, JQ825154—Primorye-52, EU816453—Primorye-69, JQ825152—Primorye-75, JQ825148—Primorye-82, EU816455—Primorye-86, JQ825149—Primorye-87, FJ906622—Primirye-89, FJ997899—Primorye-90, JQ825150—Primorye-91, HQ201303—Primorye-92, EU816454—Primorye-94, JQ825153—Primorye-183, JQ825155—Primorye-196, JQ825157—Primorye-202, JQ825158—Promorye-208, EU816450—Primorye-212, EU816451—Primorye-253, EU816452—Primorye-270, JQ825159—Primorye-274, JQ825160—Primorye-320, AY169390—Primorye-332, JQ825161—Primorye-345, JQ825162—Primorye-437, JQ825156—Primorye-739, JQ825163—Primorye-750, JQ825164—Primorye-823, JQ825144—Primorye-828, JQ825145—Primorye-895, JQ825147—Shkotovo-94, JQ825151—Spassk-72, and GU121642—Svetlogorie. The sequences EU816450, EU816451, EU816452 and AY169390 were published previously (Belikov et al., 2010).

The nucleotide and amino acid sequences were compared online at mafft.cbrc.jp/alignment/server/ using the program MAFFT version 7. The phylogenetic analysis of the nucleotide sequences of the analyzed complete genomes of the virus strains and the previously reported genome sequences of the Far Eastern subtype TBEV strains (Sofjin—AB062064, Oshima 5–10—AB062063, Glubinnoe—DQ862460, and Senzhang—AY182009) was performed by neighbor joining according to the evolutionary model proposed by Tamura and Nei using the TreeConW software package (Tigabu et al., 2009). The reliability of the reconstructed tree topology was assessed by bootstrapping based on 1000 pseudoreplicates.

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