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CHARACTERISTICS OF THE BAIKAL SUBTYPE OF TICK-BORNE ENCEPHALITIS VIRUS CIRCULATING IN EASTERN SIBERIA

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Background. During the study of the genetic variability of the tick-borne encephalitis virus (TBEV) in Eastern Siberia, a group of 22 strains with a unique genetic structure significantly different from all known TBEV subtypes was identified. This TBEV variant was tentatively called "group 886". Therefore, for this original TBEV variant it was necessary to study the genetic, biological properties of the "group 886" strains, clarify its TBEV taxonomic status, its range, evolutionary history, etc.
Aim. The generalization of the currently available data on genetic and biological properties of TBEV "886" group.
Materials and methods. The genetic structure of "group 886" strains was studied by the complex of molecular-genetic methods (MHNA, sequencing of fragments or the complete genome).
Results. It was shown that "group 886" strains form a separate cluster on phylogenetic tree, and the level of genetic differences from other genotypes is more than 12 %. It was defined that this TBEV variant has its own area (Irkutsk region, Republic of Buryatia, Trans-Baikal region, Northern Mongolia). Its ecological connection with all links of the transmissive chain (ixodid ticks, small mammals, human), participation in human pathology, stability and duration of circulation in the Baikal region, individual evolutionary history were proved. Some phenotypic characteristics of the "group 886" strains were considered.
Conclusion. The presented data testify to the validity of the "886 group" isolation as an independent genetic type. Taking into account the geographical distribution of this TBEV genotype, we propose to assign it the name "Baikal genotype/subtype".

Key words: *tick-born encephalitis virus (TBEV), genotype, subtype*

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ХАРАКТЕРИСТИКА БАЙКАЛЬСКОГО СУБТИПА ВИРУСА КЛЕЩЕВОГО ЭНЦЕФАЛИТА, ЦИРКУЛИРУЮЩЕГО НА ТЕРРИТОРИИ ВОСТОЧНОЙ СИБИРИ

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Введение. В ходе проведения цикла работ по исследованию генетической вариабельности вируса клещевого энцефалита (ВКЭ) на территории Восточной Сибири выявлена группа из 22 штаммов, обладающих уникальной генетической структурой, существенно отличающейся от всех известных генотипов/субтипов ВКЭ. Этот вариант ВКЭ получил предварительное название «группа 886» или генотип 5 ВКЭ. В связи с существованием оригинального варианта ВКЭ необходимо исследование генетических, биологических свойств штаммов «группы 886», уточнение его таксономического статуса ВКЭ, ареала, эволюционной истории и т.д. Цель. Обобщение всего имеющегося на сегодняшний день материала о генетических, биологических свойствах ВКЭ «группы 886».
Материалы и методы. С помощью комплекса молекулярно-генетических методов (МГНК, секвенирование фрагментов или полного генома штаммов ВКЭ) исследована генетическая структура штаммов «группы 886». Результаты. Показано, что штаммы «группы 886» формируют отдельный кластер на филогенетическом древе, уровень генетических отличий от других генотипов составляет более 12 %. Установлено, что данный вариант ВКЭ имеет собственный ареал (Иркутская область, Республика Бурятия, Забайкальский край, Северная Монголия). Доказаны его экологическая связь со всеми звеньями трансмиссивной цепи (иксодовые клещи, мелкие млекопитающие, человек), участие в патологии человека, стабильность и длительность циркуляции на территории Байкальского региона, индивидуальная эволюционная история. Рассмотрены некоторые фенотипические характеристики штаммов «группы 886», свидетельствующие о высоком патогенном потенциале этого варианта ВКЭ и его способности приспосабливаться к циркуляции в разных биоценозах и в различных ландшафтно-географических зонах.
Выводы. Представленные данные свидетельствуют в пользу правомерности аттестации «группы 886» в качестве самостоятельного генетического типа – генотипа 5. Учитывая географическое распространение данного генотипа ВКЭ, мы предлагаем присвоить ему название «Байкальский генотип/субтип».

Ключевые слова: *вирус клещевого энцефалита, генотип, субтип*

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INTRODUCTION

In the XXI century tick-borne encephalitis (TBE) remains the most distributed severe natural foci infection transmitted by *Ixodes* tick bite. The causative agent of this infection is a tick-born encephalitis virus (TBEV). According to the modern classification, it belongs to the group of mammal viruses transmitted by ticks, and is a member of the genus *Flavivirus* of the family *Flaviviridae* [9]. Based on phylogenetic analysis of E protein, the TBEV has been classified into three subtypes, namely European (Eu-TBEV), Far-Eastern (FE-TBEV), and Siberian (Sib-TBEV) [10–12].

The circulation of three TBE virus subtypes in Eastern Siberia with Sib-TBEV domination was identified by the study. Besides, the unique strains (886-84 and 178-79), which possess the different genetic structure compare to other three TBEV subtypes have been found on this territory [5]. For the first time the irregularity of 886-84 strain was found when its serological properties were investigated. A.G. Trukhina suggested that this strain takes intermediate place between two TBEV serotypes – East Siberian and Far-Eastern and shows properties of both serotypes [8].

Then, 886-84 strain was described as a representative of an independent genotype, according to criteria, developed by our team comparing the difference level of 29 strains isolated on different territories of TBEV area [4]. As a model, we used the fragment of E protein gene (positions 567 to 727). It was found that 886-84 strain amino acid sequences of this fragment (fragment of E protein 53 nt) has Leu in position 204 as genotype 3 and Asp in position 234 as genotypes 1 and 2 [5]. At that time we did not have any homological isolates and decided than new data are necessary to separate this TBEV strain into an independent genotype.

Now, by the molecular hybridization of nucleic acids method (MHNA) with genotype specific probes, gene sequencing of complete virus genome and its fragments we identified the group of 22 strains which have homology to 886-84 strain that was conditionally defined as “group 886” [2]. The results confirm the validity of “group 886” certification as a separate TBEV subtype.

The aim of our study was to summarize the currently available data on TBEV “886 group”.

MATERIALS AND METHODS

TBE virus. 22 TBEV strains from the collection of Federal State Public Scientific Institution «Scientific Centre for Family Health and Human Reproduction Problems» (Collection of strains of tick-borne encephalitis virus, ckp-rf.ru/ckp/478258/) were investigated in the study. By MHNA genotyping and full genome and fragments sequencing they were classified as “group 886” strains. Detailed strain information is presented in Table 1.

Strain genotyping. We used MHNA with three panels in 40 deoxyoligonucleic probes complemented to fragments of 10 genes of different TBEV subtypes. The probe description and their localization in TBEV genome was presented in the study by Demina et al. [2].

Table 1

Information concerning "group 886" strains of TBE virus, isolated on the Eastern Siberia territory

Strain No.	The year of isolation	Isolation source	The location of material collection
886-84	1984	*Myodes rutilus*	Irkutsk region, Ekhirit-Bulagatskiy district
258-83	1983	*I. persulcatus*	Buryat Republic, Bichurskiy district
265-83	1983	*I. persulcatus*	Buryat Republic, Bichurskiy district
287-83	1983	*I. persulcatus*	Buryat Republic, Bichurskiy district
711-84	1984	*M. rufocanus*	Buryat Republic, Barguzinskiy district
712-89	1989	*I. persulcatus*	Transbaikalia, Krasnochikoyskiy district
740-84	1984	*M. rufocanus*	Buryat Republic, Bichurskiy district
768-89	1989	*I. persulcatus*	Buryat Republic, Bichurskiy district
772-89	1989	*I. persulcatus*	Buryat Republic, Bichurskiy district
774-89	1989	*I. persulcatus*	Buryat Republic, Bichurskiy district
780-89	1989	*I. persulcatus*	Buryat Republic, Bichurskiy district
803-89	1989	*I. persulcatus*	Buryat Republic, Bichurskiy district
418-90	1990	*I. persulcatus*	Transbaikalia, Krasnochikoyskiy district
590-90	1990	*I. persulcatus*	Buryat Republic, Bichurskiy district
606-90	1990	*I. persulcatus*	Buryat Republic, Bichurskiy district
608-90	1990	*I. persulcatus*	Buryat Republic, Bichurskiy district
617-90	1990	*I. persulcatus*	Buryat Republic, Bichurskiy district
636-90	1990	*I. persulcatus*	Buryat Republic, Bichurskiy district
691-90	1990	*I. persulcatus*	Buryat Republic, Bichurskiy district
703-90	1990	*I. persulcatus*	Buryat Republic, Bichurskiy district
733-90	1990	*I. persulcatus*	Transbaikalia, Krasnochikoyskiy district
742-90	1990	*I. persulcatus*	Transbaikalia, Krasnochikoyskiy district

The total RNA extraction from infected mice brain or porcine embryo kidney cells, applying RNA onto kapron or cellulose nitrate filters, and hybridization with probes were performed by the common methods [14].

The amplification was carried out with primers corresponded to fragment of 5'- UTR, to C- prM-E-NS1 genes, E gene, E and NS1 gene fragments, synthesized in the Institute of Chemical Biology and Fundamental Medicine SB RAS (Novosibirsk). RT-PCR was performed according to the company "Biosan" (Novosibirsk) protocol.

The sequence analysis of PCR products was carried out with BigDye Terminators Cycle Sequencing Kit v.3.1 (Applied Biosystems, USA) on the DNA sequencer ABI 310 (Applied Biosystems, USA) in the DNA Sequencing Center SB RAS, Novosibirsk. The obtained data were analyzed by Mega 5.0 program [19]. We used sequences of different TBEV strains gene fragments, belonging to different genetic subtypes from GenBank database as a material for comparison. We used BLAST program (http://www.ncbi.nlm.nih.gov/blast/) to search for homology of nucleic sequences with already known fragments of TBEV genome.

The nucleic sequences of "group 866" strains, obtained during the study have been deposited into international electronic database GenBank, access numbers are EF469662, EU878281-EU878283, JN936341, JN936347, JN936349-JN936350, JN936353-JN936355.

The sequencing of full genome of 886-84 strain and fragments of 606-90 and 608-90 strains have been performed by L.S. Karan et al., in Central Research Institute of Epidemiology of Rospotrebnadzor RF, Moscow.

RESULTS AND DISCUSSION

Comparing the complete strain 886-84 genome (EF469662) with TBEV sequences available in GenBank has shown, that it develops an independent branch and does not cluster with any of three main subtypes (Fig. 1) and by the nucleic substitution level is close to the species separation border [6] (Table 2).

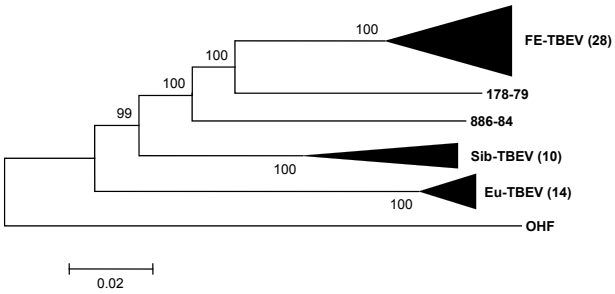

Fig. 1. Phylogenetic tree demonstrates the genetic relation level of 54 TBEV strain obtained on the base of polyprotein coding region (1042 nt). FE-TBEV cluster – Sofjin [18] X07755, AB022703, AB001026, DQ989336, AY182009, AY217093, JF316707, JF316708, FJ997899, EU816450-EU816455, AY169390, FJ906622, GQ228395, FJ402885, FJ402886, DQ862460, GU121642, HQ201303, HQ901367, HQ901366, HM859894, HM859895, JN003205; Eu-TBEV cluster – TEU27495, TEU27491, TEU39292, AF091010, EU106868, DQ401140, GV266392, HM535610, HM535611, HM120875, GU183379-GU183381, GU183383; Sib-TBEV cluster – L40361, AF527415, DQ486861, FJ968751, JN003206-JN003209, GU183382, GU183384.

Table 2

Nucleic and amino acid substitution level between TBEV subtypes and strain 886-84 (%) (polyprotein coding region 1042 nt)

Nucleic substitution level (%) (coding region of polyprotein, 10242 nt)			
	FE-TBEV	**Eu-TBEV**	**Sib-TBEV**
FE-TBEV	4,3		
Eu-TBEV	16,4	2,3	
Sib-TBEV	14,4	15,2	5,4
178-79	11,0	16,0	14,1
886-84	12,5	15,6	13,7
Amino acid substitution level (%) (complete amino acid sequence of polyprotein, 3414 ar.)			
FE-TBEV	1,3		
Eu-TBEV	6,9	0,9	
Sib-TBEV	5,3	6,2	1,9
178-79	3,1	6,1	5,2
886-84	3,9	6,0	4,2

Comments: The level of nucleic and amino acid substitutions is shown in grey color.

The analysis of complete amino acid sequence of strain 886-84 confirmed that its genetic structure is unique "mixture" of sequences common for subtypes FE-TBEV, Eu-TBEV and Sib-TBEV. For example, in a set of 22 positions which clear differentiate all known TBEV strains into three subtypes, the substitutions of unique amino acids (alanine (A) in position C-108, serine (S) - NS2A-127 and glycine (G) – NS3-258) with amino acids, common for main subtypes were detected for strain 886-84 [3] (Table 3).

Thirty unique substitutions were detected in strain 886-84, which were "subtype-specific" for "group 886".

Now, using MHNA and sequencing method we have found a group of 22 isolates with genetic structure analogous to strain 886-84.

Our study determined specific areas for TBEV "group 886" (Fig. 2).

Strains forming this TBE virus variant were isolated from material collected in Irkutsk region, Buryat Republic and Transbaikalia. Two "group 886" strains were isolated on the territory National Park "Alkhanai" in Duldurginskiy district, Transbaikalia, *I. persulcatus* (1999) and one from *Myodes rutilus* (2010) [1].

A common feature for meant territories is presence of several landscape forms that provide rich biodiversity. Combining of forest landscapes with steppe areas is quite common for Ekhirit-Bulagatskiy district, Irkutsk region. Bichurskiy district of Buryat Republic is presented by mountain forest ecosystems as well as submountain and mount-valley areas including submount landscapes with

Table 3

Differences between TBEV strains in 22 positions, obtained by comparing of 54 polyprotein structures

Amino acid position				TBEV subtypes				
				886-84	**178-79**	**FE-TBEV**	**Eu-TBEV**	**Sib-TBEV**
C-3				R	R	G	K	R
C-108				A	V	V/L/I	I	T
E-206				L	S	S	V	L
E-317				I	T	I/T	A	T
NS1-54				N	T	T	S	N
NS1-141				S	S	S/G	Q	G
NS1-285				K	K	R	T	K
NS2A-100				S	N	N	S	G/S
NS2A-127				S	G	A	D/E	G
NS2A-174				M	M	M/V	V	I/V
NS2A-175				L	L	L	C	I/F
NS2A-225				A	T	I/V	A	T
NS3-126				I	I	I	L/I	M/T
NS3-258				G	V	V	A	M/V/A
NS3-376				V	V	I/T	A	V
NS4B-21				Q	Q	H	R/Q	Q
NS4B-28				G	G	E	S	G
NS4B-96				A	A	A/R	T	S
NS5-18	S	S	G	N	S			
NS5-297	G	G	R	E/A	G/R			
NS5-671	V	V	V/G	L	I			
NS5-832	A	A	A/V/T	M	T/A			

Comments. Each shade of grey color in the cell points on amino acid residue correspondence to one of four TBEV subtypes. Black color shows that amino acid is unique for strain 886-84.

local pinewoods and steppe-meadows. Barguzinskiy district is located from Barguzin river mouth along Barguzin river valley in mountain-forest zone. Its middle part is an "island" of steppe and forest-steppe landscapes in isolated mountain valley surrounded by mountain-forest area. Krasnochikoiskiy district, Transbaikalia, is the eastern frontier of South-Siberian mountain landscape territory. Basic components of foci territories are similar to ones on Irkutsk region and Buryat Republic south, where we observe combining of mountain-forest, forest steppe, and steppe landscapes. The landscape of National Park "Alkhanai" is also very diverse: steppes, meadows, forests, and rocky mounts. The National Park location on the border of Eurasia forests and Dauria steppes has a special biospheric value and significant biodiversity as a result of flora and fauna interpenetration. Bulganskiy aimak, Mongolia, located in Selenga river basin is characterized by forest-steppe, steppe, dry steppe zones and river valleys.

We obtained data concerning the ecological connection for "group 886" strains with all elements of transmissive chain. Strains 258-83, 265-83, 287-83, 712-89, 768-89, 772-89, 774-89, 780-89, 803-89, 418-90, 590-90, 606-90, 608-90, 617-90, 636-90, 691-90, 703-90, 733-90 and 742-90 were isolated from *I. persulcatus* ticks, 711-84 and 740-84 from *Myodes rufocanus* brain, 886-84 - from *Myodes rutilus*. Recently, a case of meningoencephalitis with lethal outcome was described in Mongolia, caused by isolate with genetic structure possessing the level of homology 98.5 % to strain 886-84. Infection took place in Bulganskiy aimak bordering from south four foci where «group 886» strains were isolated from collected material. The patient was hospitalized on 11th day after the tick bite with diagnosis "meningoencephalitis" and died on 11th day of the disease. Presence of TBEV RNA in macromyelon samples, in core and meninx vasculosa shows that the multilevel localization of lesions and is typical to the most severe forms of acute TBE which results in lethal outcome or disability [15]. Our previous studies to determine the virulence of «group 886» strains for laboratory animals have confirmed that this TBEV variant has a high pathogenic potential. Six of ten examined «group 886» strains had high invasive properties, that means they are able to overcome the blood-brain barrier, penetrate into CNS and propagate in it [16]. Having studied genetic markers connected to intracellular reproduction we showed that «group 886» strains have high adaptive ability and, consequently can easily adapt to circulation in different biocenoses and in variety of landscape-geographical zones [17].

The data obtained in this study raise the question of taxonomic status of TBEV "group 886". It was shown that the level of genetic differences of TBEV "group 886" from other three subtypes is more than 12 %. In a set of 22 positions, which clearly differentiate all known TBEV strains into three subtypes, the substitution of the unique amino acids (alanine (A) in position C-108, serine (S) – NS2A-127 and glycine (G) – NS3-258) with amino acids, common for main subtypes was detected for strain 886-84. These data testify to the validity of the "group 886" certification as an independent TBEV genetic type.

The Baikal subtype of TBEV has its own evolutionary history. According to S.Y. Kovalev, a group of 886-like strains is the youngest one among Siberian strains, and its approximate age is 99 (95 %, 81-118 years) [16]. According to his hypothesis, Transbaikalia (including Buryatia, northern Mongolia, the Trans-Baikal and the Irkutsk regions) is sympatric zone of two *I. persulcatus* tick races – western (area up to Baikal Lake) and eastern (Far East), and the place of occurrence of ticks' interracial hybrids. In his opinion, a transitional form between the Far Eastern and Siberian subtypes of the virus can exist in such ticks' interracial hybrids, and "group 886" strains are

Fig. 2. TBEV "group 886" area of habitat.

considered to be such transition point. So, the conclusion about the eligibility of classifying of "group 886" as an independent subtype was made [16]. It was pointed out that, despite the presence of Leu at position 206 of E protein, as in FE-TBEV strains, the independent evolutionary origin of the "group 886" from FE-TBEV testifies to the need of its isolation into an individual subtype.

Reconstructing the evolution of the TBE complex viruses, D.M. Heinze et al. concluded that in the eastern group of TBEV, a divergence of the Sib-TBEV has occurred about 2,400 years ago (95%, 1300-3800), then the Irkutsk isolates have separated: 886-84 – 1800 years ago (95%, 1400-3800) and 178-79 – 1400 years ago (95%, 1200-2800), and then the FE-TBEV has divided [13].

Despite the differences in age characteristics, obtained by the authors during the reconstruction of TBEV evolutionary history, both these and other researchers consider the origin of the Baikal subtype to be individual.

However, in order to declare a new, independent TBEV subtype, it is necessary to take into account a number of criteria formulated earlier by V.V. Pogodina. In particular, this includes: 1) the broadness of the habitat range; 2) the stability of virus circulation, confirmed by repeated isolation of strains; 3) established role in the etiology of manifest forms of the disease; 4) the effect of strains on the formation of population immunity; 5) insufficient effectiveness of standard diagnostic and prophylactic tools [7].

For example, recently the researchers from China described a new subtype of TBEV, which they called the "Himalayan" [9]. Two strains of this TBEV subtype (Him-TBEV) were isolated as a result of study of 200 samples from the respiratory tract of the wild rodent *Marmota himalayanain*, which inhabits the Tibetan plateau in China. Phylogenetic analysis of the E gene and complete genome sequences demonstrated that the Him-TBEV strains form an independent branch separated from FE-TBEV, Eu-TBEV and Sib-TBEV. The nomenclature of Him-TBEV as a new subtype was also supported by comparative analysis using nucleotide and amino acid sequences of E protein and polyprotein [9]. For E protein, the Him-TBEV showed 82.6-84.6 % nucleotide identities and 92.7-95.0 % amino acid identities with other three subtypes. For polyprotein, the Him-TBEV showed 83.5-85.2% nucleotide identities and 92.6-94.2% amino acids identities with other three subtypes. Furthermore, of 69 amino acid substitutions profiles detected in complete polyprotein of 112 strains of TBEV, Him-TBEV subtype displayed unique amino acids in the 36 positions. Notably, for the subtype-specific amino acid position 206 of E protein, Him-TBEV shared the Val with Eu-TBEV, but differed from FE-TBEV and Sib-TBEV. The evolutionary analysis with BEAST suggested that Him-TBEV diverged from other subtypes of eastern TBEV group about 2469 years ago [9]. According to this study, the divergence time of the "group 886" from TBEV eastern group was approximately 1819 years ago, which matches data previously obtained by D.M. Heinze et al. [9, 13].

In our opinion, both the strain 178-79, previously described by us as an independent subtype, and the "Himalayan" subtype, cannot yet be recognized as independent subtypes, until they don't fit the criteria necessary for such conclusion. Strains of these subtypes are single, and their broadness of the habitat range, the role in human pathology, the stability of circulation, etc. are still unknown. Currently, the only TBEV subtype described by us – "group 886" – fits all these criteria.

The case of meningoencephalitis with lethal outcome described in Bulganskiy aimak, Mongolia, caused by isolate with genetic structure, homologous to 886-84 strain demonstrates that this TBEV variant may play the role in human infectious pathology, while the "886-group" strains isolation during long period (since 1983 to 2010) confirms stability of its circulation in Eastern Siberia.

Thus, based on presented data, the strains of the "group 886" could possibly be declared as a new independent subtype. Taking into account the geographical distribution of this subtype, we propose to assign it the name "Baikal subtype".

CONCLUSION

1. New data concerning original TBE virus variant circulating on the territory of Eastern Siberia have been obtained. We showed the unique genetic structure of «group 886» strains that is a "mixture" of amino acid sequences common for FE-TBEV, Eu-TBEV and Sib-TBEV.
2. This TBEV variant can be considered as an independent TBEV subtype (high level of genetic difference comparing to other genotypes – more than 12 %, existence of its own natural area, ecological connection with all elements of transmissive chain, role in infectious pathology, stability and duration circulation in nature).
3. Ability to cause focal forms of tick-born encephalitis with lethal outcome and laboratory results of virulence level assessment testify the high pathogenic potential of "group 886" TBEV strains, and independent evolutionary origin.
4. The presented data allow isolating the "group 886" strains as a new independent subtype. Taking into account the geographical distribution of this TBEV genotype, we propose to assign it the name "Baikal subtype".

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