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Genetic Studies of Tick-Borne Encephalitis Virus Strains from Western and Eastern Siberia

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1. Introduction

Tick-borne encephalitis is a zoonotic infection of a central nervous system whose clinical course is often severe, even fatal, and complications (such as paralysis) are frequent [Zlobin and Lvov, 2008]. It is caused by tick-borne encephalitis virus (TBEV) belonging to the mammalian tick-borne flaviviruses group in the genus *Flavivirus* (*Flaviviridae*). The group includes several species found in geographically distant regions of Eurasia and North America. Evolutionary and genetic relationships among these viruses as well as among TBEV strains proper are not clear, which results in the contradiction of classification schemes suggested by different authors. There is a widely shared opinion that three antigenic TBEV subtypes correspond to three major TBEV genotypes, the Far Eastern (TBEV-FE, with Sofjin strain as a prototype), European (TBEV-Eu, prototype Neudoerfl strain), and Siberian or Ural-Siberian (TBEV-Sib, prototypes Vasilchenko and Zausaev strains) (Fig. 1) [Ecker et al., 1999; Hayasaka et al., 1999, 2001; Zlobin et al., 2001a, 2003; Pogodina et al., 2002, 2004].

In 2001 as a result of studying TBEV genetic variability the existence of six genotypes of the virus was supposed on the basis of homology analysis of a small part of the E gene (160 nt) of 34 strains [Zlobin et al., 2001a,b]. Three of them were accepted as the main ones [Zlobin et al., 2003]. The Turkish strain described earlier as a separate strain in the TBEV virus subgroup [Gao et al., 1993] was included in the genotype 6 together with the strain Vergina [described as a strain of TBEV by Pogodina et al., 1993] and classified as the variant of Louping ill virus (LIV, the Scottish sheep encephalomyelitis virus) [Heinz et al., 2000]. Genotypes 4 and 5 were represented by single strains, 178-79 and 886-84. These strains were isolated in the Irkutsk region and met the criteria for classification into independent genotypes according to the degree of genomic differences [Zlobin et al., 2001a]. Then, the complete genome sequences of 178-79 and 886-84 strains have been determined [Karan' et al., 2007a,b]. Phylogenetic analysis has revealed that strain 178-79 to the greater degree and

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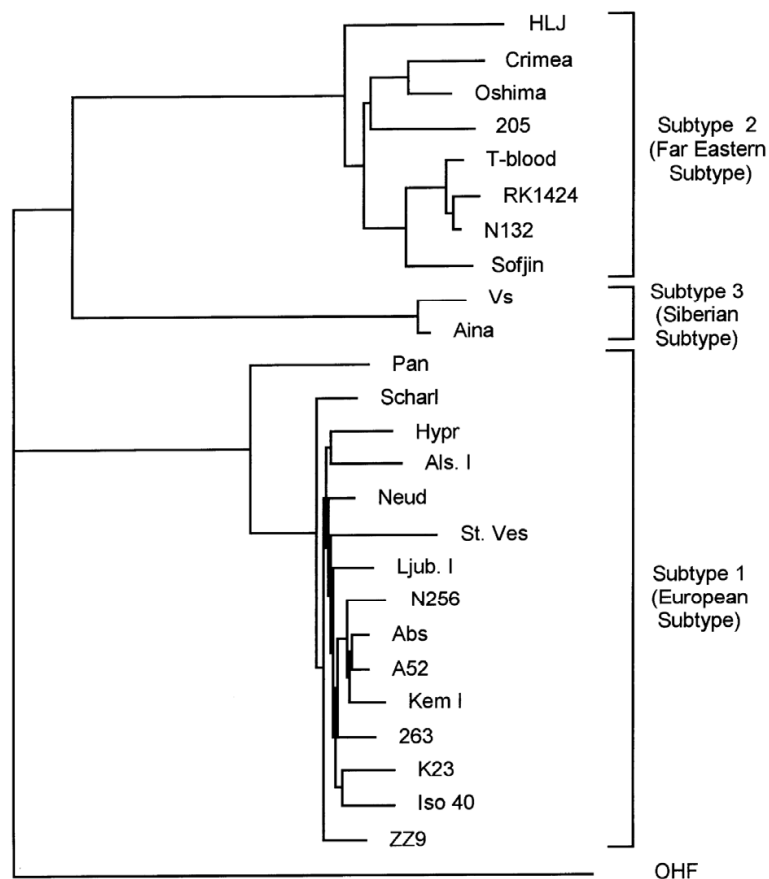


Fig. 1. Phylogenetic tree illustrating the genetic relationship of TBE virus isolates (generated by the DNADIST/FITCH program). The tree was rooted using Omsk haemorrhagic fever (OHF) virus as the outgroup virus [Ecker et al., 1999]

Differences in coding genome region (10,242 nt)				
	Genotype 1	Genotype 2	Genotype 3	178-79
Genotype 1	5,2			
Genotype 2	16,5	2,6		
Genotype 3	14,6	15,4	6,6	
178-79	11,2	16,2	14,3	
886-84	12,6	15,6	13,7	11,8
Differences in full-length polyprotein sequences (3,414 aa)				
Genotype 1	1,5			
Genotype 2	6,8	1,2		
Genotype 3	5,0	5,9	1,7	
178-79	3,2	6,3	4,6	
886-84	3,8	6,2	4,3	3,2

Five strains of genotype 1 (DQ989336, AB062064, AB062063, AY182009, DQ862460) and three for genotype 2 (U27495, U27491, U39292) and 3 (L40361, AF527415, DQ486861) were used for analysis. The substitutions levels within the genotypes are marked with gray background.

Table 1. The Differences in Level of Nucleotide and Amino Acid Sequences for 13 TBEV Strains According to Karan' et al. [2007b] (%)

strain 886-84 to the lesser degree adjoined the TBEV-FE genotype and amino acids alternation observed in the polyprotein structure were typical for two and three genotypes within one gene (Table 1).

The last attempt of re-classification of TBEV was suggested by Grard et al. [2007]. During the analysis of full-length genomes of Spanish and Turkish sheep encephalitis viruses they made the conclusion that it could be possible to divide TBEV into four genotypes: Western, Eastern, Turkish sheep encephalitis and Scottish sheep encephalitis (Fig.2). According to the proposed classification TBEV-Sib was included into the Eastern genotype. However, genotype 3 (TBEV-Sib) widely spread in Russia demonstrates the high level of genetic differences from genotypes 1 (TBEV-FE) and 2 (TBEV-Eu) including some type-specific markers possessed by a large number of circulating variants.

Siberia is the part of Russia located between European part and Far-Eastern part and can be divided to Western Siberia and Eastern Siberia (Fig. 3). The aim of the study was the molecular-genetic investigations and genotyping of TBEV strains and isolates samples collected at different regions of Western and Eastern Siberia.

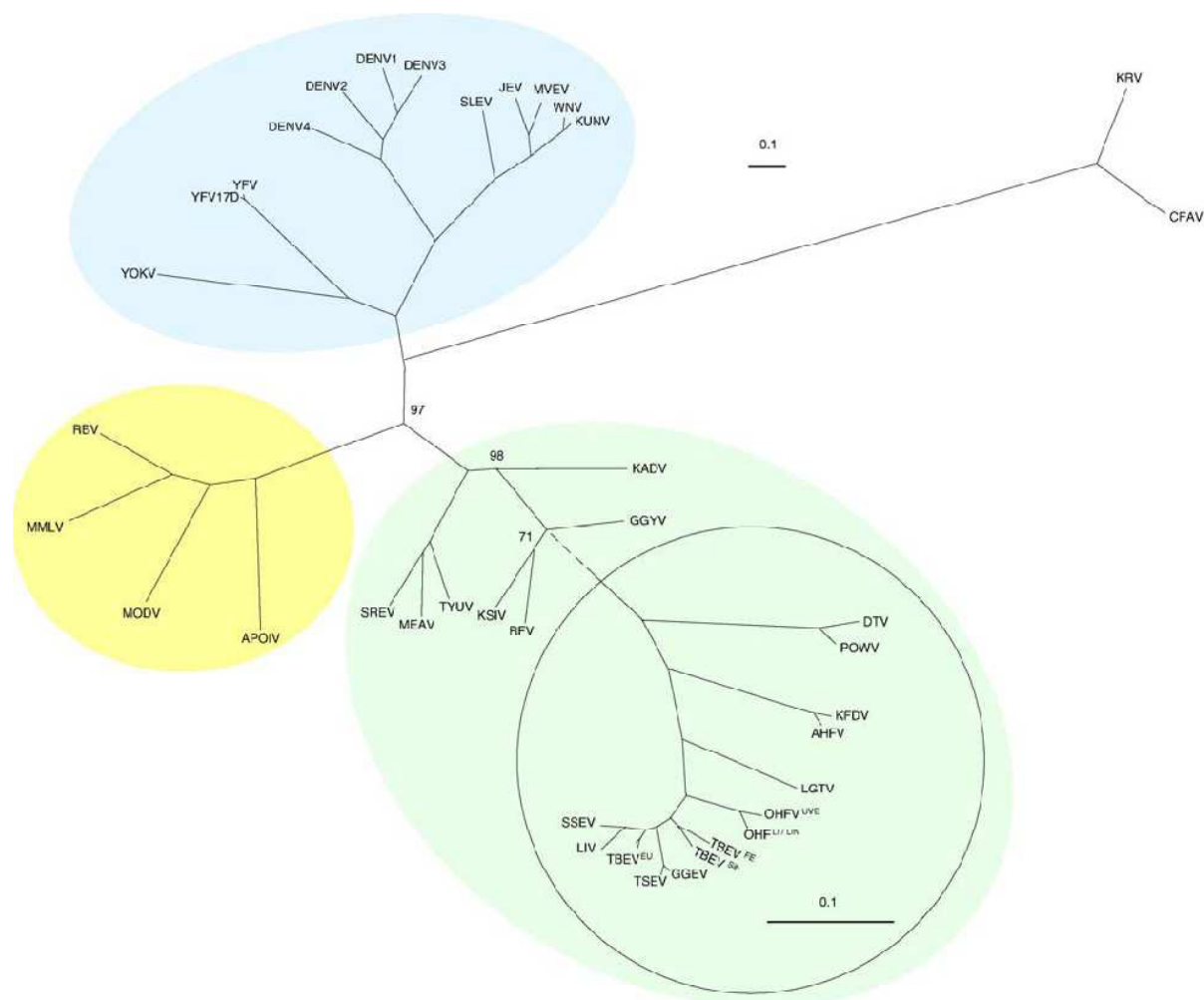


Fig. 2. Phylogenetic analysis based on complete polyprotein sequences [Grard et al., 2007] Phylogenetic reconstruction was performed using the maximum likelihood method. The tick-borne flavivirus group is highlighted in green, the mosquito-borne flavivirus group in blue and the no-known vector flavivirus group in yellow



Fig. 3. Location of different parts of the Russia territory

2. Genotyping by molecular hybridization of nucleic acids test with genotype-specific deoxyoligonucleotide probes

2.1 Design of genotype-specific deoxyoligonucleotide probes

The experiments with TBEV genotype-specific probes performed earlier [Zlobin et al., 2001c] revealed the following important points: 1) the differentiation of TBEV strains by using three genotype-specific probes yields an absolute answer and thus does not allow to type strains that have mutation(s) within the annealing sites; 2) an overlapping region of eight conserved nucleotides shared by all TBEV genotypes presented in the probes can cause nonspecific hybridization; and 3) the annealing temperatures of probes varying from 51 to 67°C may cause inconveniences in performing serial experiments. Thus, the following conclusions have been made. Firstly, simultaneous use of genotype-specific probes targeting all TBEV genes (instead of one) will allow to eliminate the influence of single mutations on the result of genotyping and to perform more detailed genetic analysis of each strain. Secondly, the structure in which the target nucleotides are located evenly (the distances that are not more than seven nucleotides are preferable) will react more reliably as a genotype-specific probe. Thirdly, for simultaneous analysis of a large number of TBEV strains the probes with annealing temperatures of 45–47°C should be selected to achieve a highly specific reaction of each probe.

2.2 Viruses

In this study 189 TBEV strains from the collection of Institute of Epidemiology and Microbiology, SCFHHRP ESD RAMS (Irkutsk, Russia) were used. The collection contained

165 strains from Eastern Siberia (Chita region: 8, Buryatiya Republic: 54, Irkutsk region: 88, Krasnoyarsk region and Khakassia Republic: 15) and 24 strains from Western Siberia. The strains were isolated from different biological sources: from Ixodes genus ticks: 148, Dermacentor genus ticks: 2, rodents: 24, insectivorous: 1, birds: 1, from cow milk: 2 and 11 strains were isolated from clinical samples, blood, and medullispinal fluid of patients with tick-borne encephalitis (Table 2). Strains were isolated and cultivated by intracerebral infection of 2-day newborn laboratory mice and then preserved in freeze-dried state. The most strains were lyophilized on 3–7 passages. To accumulate the virus the suspension of freeze-dried strains was prepared with dilution 10^{-3} in Eagle's medium, 2% bovine serum, and antibiotics (200 U/ml). Then suckling nonlinear mice (30 μ l intracerebrally) or transplantable cell culture (porcine embryo kidney cells) was infected. The gathering of the virus culture was followed by cell lysis. Mouse autopsies were carried out 5–7 days after infection at the peak of disease. Cultural fluid was centrifuged for detritus removal (3,000 rpm/min for 30 min) and then virus was precipitated with polyethylene glycol according to Mahy [1985].

2.3 Isolation of total RNA and hybridization

RNA from cultivated virus and total cellular RNA from cerebral suspensions were isolated by the method of phenol extraction [Herrington and McGee, 1992]. RNA was spotted on kapron or cellulose nitrate filters (2 mkg/dot of total RNA from mice brain tissue or 1 ng/dot of RNA isolated from the cell culture). Radioactive labeling, hybridization, and detection of hybridized probes were carried out as described [Herrington and McGee, 1992]. Hybridization with all probes was carried out at 45–47°C.

2.4 Probe selection

The TBEV genome sites most suitable for genotyping were selected on the basis of the analysis of TBEV sequences taking into account the requirements for primer design [Innis and Gelfand, 1990] and the previously obtained experience on molecular hybridization [Zlobin et al., 1992, 2001c]. Twelve regions were selected for each of the three genotypes. The probes were targeted to all 10 genes of TBEV: one probe for genes M, C, NS1, NS2A, NS2B, NS3, NS4A, NS4B and two probes for genes E and NS5 (Fig. 4).

There were also probes targeting the E protein-encoding sequences for two subgenotypes of Siberian genotype ("Vasilchenko" and "Zausaev") and the E protein-encoding sequence of strain 178-79. Notably, the targets for three last probes are localized on the E gene since it was best studied and the largest number of sequences was known for this gene. For example, the target sequence specific for the isolate 178-79 was selected based on the 160 nt long region (encoding aa residues 190–242) of 90 TBEV strains. The majority of probes were designed on the basis of the comparison of 10 full-length TBEV genomes. The TBEV species-specific probe sh5 (specific for TBEV) has been described earlier [Shamanin et al., 1990]. The proposed panel includes 40 probes targeting all 10 genes of TBEV (Table 3). Among them, 1 probe (sh5) is species specific, 36 probes are genotype specific (12 probes for each of the three main genotypes), 2 probes (designated as 3a and 3b) are subgenotype specific, differentiating subgenotypes "Vasilchenko" and "Zausaev" of Siberian genotype, and 1 probe, based on the TBEV strain 178-79, is strain specific (for genotype 4).

Region	Isolation source	Isolation year/amount of strains	Strains total	Genotype				
				1	2	3	4	5
Eastern Siberia, Russia	Clinical samples	1963/1, 1979/2, 1982/1, 1986/1, 1998/1, 2003/1, 2004/2, 2005/1	10	1	1	8	-	-
	Cow milk	1971/1, 1979/1	2	1	-	1	-	-
	Rodents	1971/2, 1975/1, 1981/5, 1984/5, 1987/4, 1995/5, 2002/1, 2006/1	24	9	4	8	-	3
	Common shrew	1995/1	1	1	-	-	-	-
	Hazelhen	1982/1	1	1	-	-	-	-
	<i>I. persulcatus</i>	1976/1, 1979/1, 1980/5, 1983/3, 1986/ 14, 1987/7, 1988/26, 1989/5, 1990/ 34, 1991/12, 1992/11, 1993/5, 2002/ 2, 2005/1	126	6	-	112	1	7
	<i>D. nuttalli</i>	1986/1	1	-	-	1	-	-
Total			165	19	5	130	1	10
Western Siberia, Russia	Clinical samples	1961/1	1	-	-	1	-	-
	<i>I. persulcatus</i>	1985/1, 1986/21	22	3	5	14	-	-
	<i>D. pictus</i>	1986/1	1	-	-	1	-	-
Total			24	3	5	16	-	-

Table 2. Summary of TBEV isolates and strains From Western and Eastern Siberia

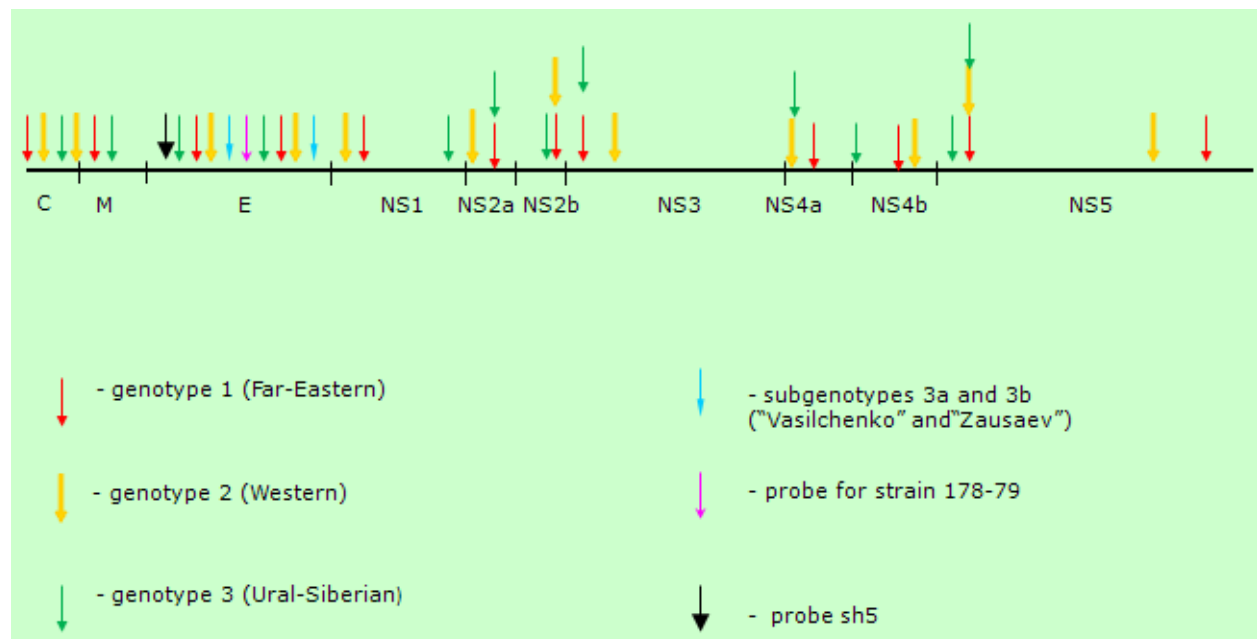


Fig. 4. The arrangement of desoxyoligonucleotide probes on TBEV genome

2.5 Testing of the probes

In the course of testing all 189 TBEV isolates have been divided into five groups corresponding to five genotypes (Tables 2 and 4). It was stated that at the territory of Eastern Siberia (165 strains) 19 strains (11.5%) belong to genotype 1 (Far Eastern), 5 strains (3.0%) belong to genotype 2 (European), and 130 strains (78.8%) belong to genotype 3 (Ural-Siberian or Siberian). The fourth group (genotype 4) included only one strain, 178-79 (0.6%). The fifth group ("group 886" or genotype 5) consisted of 10 strains (6.1%), and one of them (886-84 strain) characterized earlier [Zlobin et al., 2001a,b] was considered to be unique. As for Western Siberia territory (24 tested strains) it was demonstrated that 3 strains belong to Far-Eastern genotype (12.5%), 5 strains – to European genotype (20.8%) and 16 strains – to Siberian genotype (66.7%). None of the strains of 4th or 5th genotypes were found.

Analysis of the efficiency of the genotype-specific probes (Table 4) revealed that (i) with rare exceptions, these probes interacted only with the strains of the same name genotype and (ii) the probes specific to two different subgenotypes within genotype 3 reacted only with the strains of this genotype. All the strains of genotype 3 were further divided into three groups: subgenotype 3a (that reacted with the corresponding subgenotype-specific probe); subgenotype 3b (reacting with the corresponding probe); and "neither 3a nor 3b" strains reacting with neither of the subgenotype-specific probes. (iii) The probe intended for recognizing the strain 178-79 was strain specific. Constant interaction of some genotype 1 probes with RNA of genotypes 4 and 5 strains is explained by the fact that the structures of these reacting probes are complementary to the corresponding sites on the genome of these two strain groups. These cross-reactions could be avoided by means of selecting probes specific for genotype 1 only. C-1 probe reacted nonspecifically. This fact may be explained by the difference between its theoretical annealing temperature (57°C) and the temperature used in the experiments (47°C). Specific interaction of the probe NS5-2' with TBEV strain 178-79 confirmed that this strain is unique. Two probes, C-1 and M-2, cross-reacted with Omsk hemorrhagic fever virus (OHFV), most likely due to shared stretches of 8 and 14 nucleotides, respectively.

Probe code	Nucleotide sequence (5'→3')	Hybridization temperature (°C)	Location on genome*
sh5**	CACAATGCTGCCTTTTCCAAATAATCC	71	1156-1182
C-1	GGGTGCTCACTGCCTTCTTA	57	234-253
C-2	CGTCATCCCCAACAGAGTAA	55	323-342
C-3	CAGCAAGACAGTGACAAC	49	316-333
M-1	GCACTGTGGCTGCAAGTG	53	341-358
M-2	AGGCTCTTCTCCTTGATC	49	508-525
M-3	CCTTCCCGCCTTACTGTTG	55	350-368
E-1	GAGTCTCATTAGCAGCGACG	57	1290-1309
E-2	ACTATGTGTCTCGTTTGC	47	1297-1314
E-3	CAGTGGCCTTCTTCTTCG	51	1208-1225
E-1'	CCACCAGTAGAGCCAAAATC	55	2104-2123
E-2'	TCCCAATTGAACTCAGAAAGC	55	2123-2143
E-3'	AGTCTCTCGATGCCCTTCC	55	2060-2078
NS1-1	TCTTTCCCCTTCTTTAGC	47	2643-2660
NS1-2	TAGTCAGTGGGGTCAAAC	49	2604-2621
NS1-3	AAGAACCACAACGCCTC	47	3497-3513
NS2A-1	CCTCGAATCAGTTTCCAG	49	3939-3956
NS2A-2	CTGGAGTAGCATCAAGG	47	3614-3630
NS2A-3	TCCCCTGATCAATCTCC	47	3941-3957
NS2B-1	ATCTTCGAGCCGTTCTC	47	4452-4468
NS2B-2	TCAGACCTTCGGGATGA	47	4456-4472
NS2B-3	CAAGCCCAGCAAGTAAC	47	4368-4384
NS3-1	CACTGCCCTATCCTCCTA	55	4836-4854
NS3-2	TTTTAGGCCATTTCCGTATAGC	57	4920-4941
NS3-3	GTATTGCTCCCATCTTTCTCC	57	4835-4855
NS4A-1	ACCAAACGACGCCAAGAG	51	6521-6538
NS4A-2	CATCTCAACCATAGTCAG	47	6484-6501
NS4A-3	TCACCTCCACCATCGTGAG	55	6484-6502
NS4B-1	TCCCTCTCAAATGGATTG	51	7242-7260
NS4B-2	ATGAGGCACAACACTGTG	49	7308-7325
NS4B-3	CTCTCAAGGAACCCCATC	51	6783-6800
NS5-1	CTCTCCTGAGGAGTTCC	49	7656-7672
NS5-2	TCCTCTTCTGAGCAACTC	49	7657-7674
NS5-3	CCTTCCTTAGCAGCTCT	47	7656-7672
NS5-1'	TTCGAGAAGCCAACCGAATG	55	9622-9641
NS5-2'	GTGGTTGCCAATTGTTG	47	9219-9236
NS5-3'	CAGCTGTTCACTCTCTGT	49	7572-7589
E-3a	ACAATCCGCAGTGATTGC	49	1145-1162
E-3b	AAGGCGCCACCGAGAAC	53	2158-2174
E-4	TACCCCTTCGTGTCTCC	49	1520-1536

*Numeration is done for the coding region. **Probe sh5 is described earlier [Shamanin et al., 1990].

Table 3. Genotype-specific deoxyoligonucleotide probes and their respective targets on TBEV genome

Probe code	Strains of genotype 1 (Far Eastern subtype)		genotype 4 178-79		genotype 5 "Group 886"		Strains of genotype 2 (European subtype)		Strains of genotype 3 (Siberian subtype)	
	P/N*	%**	P/N	%	P/N	%	P/N	%	P/N*	%
sh5	17/22	77	1/1	100	6/10	60	8/10	80	137/144	95
C-1	20/22	91	1/1	100	10/10	100	0/10	0	89/146	61
M-1	20/22	91	1/1	100	0/10	0	0/10	0	1/146	1
E-1	19/22	86	1/1	100	0/10	0	0/10	0	2/146	1
E-1'	20/22	91	0/1	0	0/10	0	0/10	0	1/146	1
NS1-1	6/22	32	1/1	100	10/10	100	0/10	0	3/146	2
NS2A-1	20/22	91	0/1	0	0/10	0	0/10	0	1/146	1
NS2B-1	20/22	91	1/1	100	0/10	0	0/10	0	4/146	3
NS3-1	21/22	95	1/1	100	10/10	100	0/10	0	22/146	15
NS4A-1	16/22	72	0/1	0	0/10	0	0/10	0	0/146	0
NS4B-1	19/22	86	0/1	0	0/10	0	0/10	0	2/146	1
NS5-1	9/22	41	0/1	0	0/10	0	0/10	0	1/146	1
NS5-1'	15/22	68	0/1	0	0/10	0	1/10	10	11/146	8
C-2	0/22	0	0/1	0	0/10	0	10/10	100	0/146	0
M-2	0/22	0	0/1	0	0/10	0	10/10	100	12/146	8
E-2	0/22	0	0/1	0	0/10	0	10/10	100	0/146	0
E-2'	0/22	0	0/1	0	0/10	0	5/10	50	0/146	0
NS1-2	0/22	0	0/1	0	0/10	0	7/10	70	1/146	0
NS2A-2	0/22	0	0/1	0	0/10	0	9/10	90	2/146	1
NS2B-2	0/22	0	0/1	0	0/10	0	1/10	10	1/146	1
NS3-2	0/22	0	0/1	0	0/10	0	10/10	100	0/146	0
NS4A-2	1/22	5	0/1	0	0/10	0	5/10	50	1/146	1
NS4B-2	1/22	5	0/1	0	0/10	0	3/10	30	1/146	1
NS5-2	0/22	0	0/1	0	0/10	0	10/10	100	0/146	0
NS5-2'	0/22	0	1/1	100	0/10	0	10/10	100	7/146	5
C-3	0/22	0	0/1	0	0/10	0	0/10	0	15/146	10
M-3	0/22	0	0/1	0	0/10	0	0/10	0	38/146	26
E-3	0/22	0	0/1	0	0/10	0	0/10	0	96/146	66
E-3'	0/22	0	0/1	0	0/10	0	0/10	0	44/146	99
NS1-3	0/22	0	0/1	0	0/10	0	0/10	0	112/146	77
NS2A-3	1/22	5	0/1	0	0/10	0	0/10	0	80/146	55

Probe code	Strains of genotype 1 (Far Eastern subtype)		genotype 4 178-79		genotype 5 "Group 886"		Strains of genotype 2 (European subtype)		Strains of genotype 3 (Siberian subtype)	
	P/N*	%**	P/N	%	P/N	%	P/N	%	P/N*	%
NS2B-3	1/22	5	0/1	0	0/10	0	0/10	0	60/146	41
NS3-3	1/22	5	0/1	0	0/10	0	0/10	0	131/146	90
NS4A-3	0/22	0	0/1	0	0/10	0	0/10	0	71/146	49
NS4B-3	2/19	9	0/1	0	0/10	0	0/10	0	105/146	72
NS5-3	0/22	0	0/1	0	0/10	0	0/10	0	114/146	78
NS5-3'	1/22	5	0/1	0	0/10	0	1/10	10	85/146	58
E-3a	0/22	0	0/1	0	0/10	0	0/10	0	34/146	23
E-3b	0/22	0	0/1	0	0/10	0	0/10	0	30/146	21
E-4	0/22	0	1/1	100	0/10	0	0/10	0	0/146	0

1',2',3' – Probes targeting the gene of the same TBEV genotype.

*Number of positive reactions/number of tested samples. **Hybridization efficiency (%).

Table 4. Effect of hybridization of genotype-specific deoxyoligonucleotide probes with investigated TBEV strains

3. Genotyping by phylogenetic analysis approach

3.1 Viruses

In this study 1) 70 TBEV strains from the collection of Institute of Systematics and Ecology of Animals SB RAS (Novosibirsk, Russia) and 2) 54 strains from the collection of Institute of Epidemiology and Microbiology, SCFHHRP ESD RAMS (Irkutsk, Russia) were used. The first ones were isolated by bioassay method from *Ixodes persulcatus* ticks collected at the territory of Novosibirsk scientific center and its suburbs in 1981-2001 and cultivated for 2-16 passages in laboratory mice. The second ones were isolated from different source at the territory of Western and Eastern Siberia (see part 2 of the Chapter) and cultivated by intracerebral infection of 2-day newborn laboratory mice and then preserved in freeze-dried state.

3.2 Isolation of total RNA, reverse transcription (RT)-PCR and sequencing

RNA from cultivated virus and total cellular RNA from cerebral suspensions were isolated by the method of phenol extraction [Herrington and McGee, 1992]. The reverse transcription was performed with the kit "RevertaL-100" contained random hexanucleotides (Amplisense, Moscow, Russia). Amplification was done with primers corresponding to specific regions of the E gene (positions 1089-1108 and 2367-2386 the genome of TBEV Sofjin-HO strain (AB062064)) or fragments of E and NS1 genes (positions 2216-2236 and 3364-3383 or 2199-2219 and 2517-2539 on the genome of TBEV Sofjin-HO strain). The primers were synthesized in the Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the Russian Academy of Science (Novosibirsk, Russia). These primers were selected on the basis of sequences of highly conservative sections of three TBEV

genotypes (strains Sofjin, Neudoerfl, Vasilchenko, and Zausaev). One- or two-round PCR was carried out using these oligonucleotides as primers following the manufacturer's recommendations (Biosan, Novosibirsk, Russia). The direct sequencing of PCR products corresponded to E or E and NS1 genes was performed with BigDye Terminators Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA) in the Center of DNA sequencing of the Siberian Branch of the Russian Academy of Science (Novosibirsk, Russia).

3.3 Molecular-genetic analysis

The alignment of obtained sequences was performed with ClustalW program [Larkin et al., 2007]. Homology search with known TBEV genome fragments sequences was made with BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>). The sequences of the strains of different TBEV genotypes from GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) were used for comparison. Evaluation of phylogenetic interactions of TBEV based on the full-length genomes or genome fragments was implemented using programs Mega 4.0 [Tamura et al., 2007] and TreeCon [Van de Peer and De Wachter, 1994]. Sequences of the following strains were used: Sofjin [Pletnev et al., 1990], Sofjin-HO (AB062064), 205 (DQ989336), Oshima-10 (AB062063), Senzhang (AY182009), MDJ-01 (AY217093), Glubinnoe (DQ862460), Prymorye-69 (EU816453), Prymorye-86 (EU816455), Prymorye-94 (EU816454), Prymorye-212 (EU816450), Prymorye-253 (EU816451), 178-79 (EF469661), 886-84 (EF469662), Prymorye-270 (EU816452), Neudoerfl (TEU27495), 263 (TEU27491), Hypr (TEU39292), K23 (AM600965), Salem (FJ572210), Vasilchenko (L40361), Zausaev (AF527415), Ek-328 (DQ486861), and Omsk hemorrhagic fever virus (NC_005062)).

3.4 TBEV strains genotyping

The nucleotide sequences of genomes fragments with length 518 bp (207-724 nr from the beginning of E gene) obtained by RT-PCR followed by sequencing were determined for 70 TBEV strains isolated at the territory of Novosibirsk scientific center (58 strains) and Novosibirsk city suburbs (12 strains) in 1981-2001. According to the different structure models this E protein region encoded by the appropriate gene region was shown to contain I and II domains fragments [Rey et al., 1995] or E1 an E2 functionally significant domains fragments [Tsekhanovskaya et al., 1993], or A and C antigenic domains fragments [Mandl et al., 1989]. The obtained sequences were submitted into GenBank database (accession numbers EF467840-EF467846, EF469738-EF469741, EF469743, EF469744, EF469746-EF469748, EF469750, EF469751, EF469754, EF469755, EF469757, EF469760-EF469762, EF469764, EF470568-EF470575, EF470577, EF470578, EU443258-EU443260, EU443263-EU443271, EU443274, EU443275, EU443277-EU443279, EU443281-EU443284, EU836250).

For the further comparative analysis the sequences of TBEV prototype strains of Far-Eastern (Sofjin strain), Siberian (Zausaev and Vasilchenko strains) and European (Neudoerfl strain) genotypes were used. The analysis of obtained data and homology search revealed high homology level of investigated sequences with Siberian genotype TBEV strains (>92% of homology with Zausaev subgenotype and >93% of homology with Vasilchenko subgenotype) and reliable differences from TBEV strains of European and Far-Eastern genotypes (<87% and 85% of homology, respectively). But one should note that 100% homology level wasn't found between obtained sequences and sequences of flavivirus genome fragments from Genbank database.

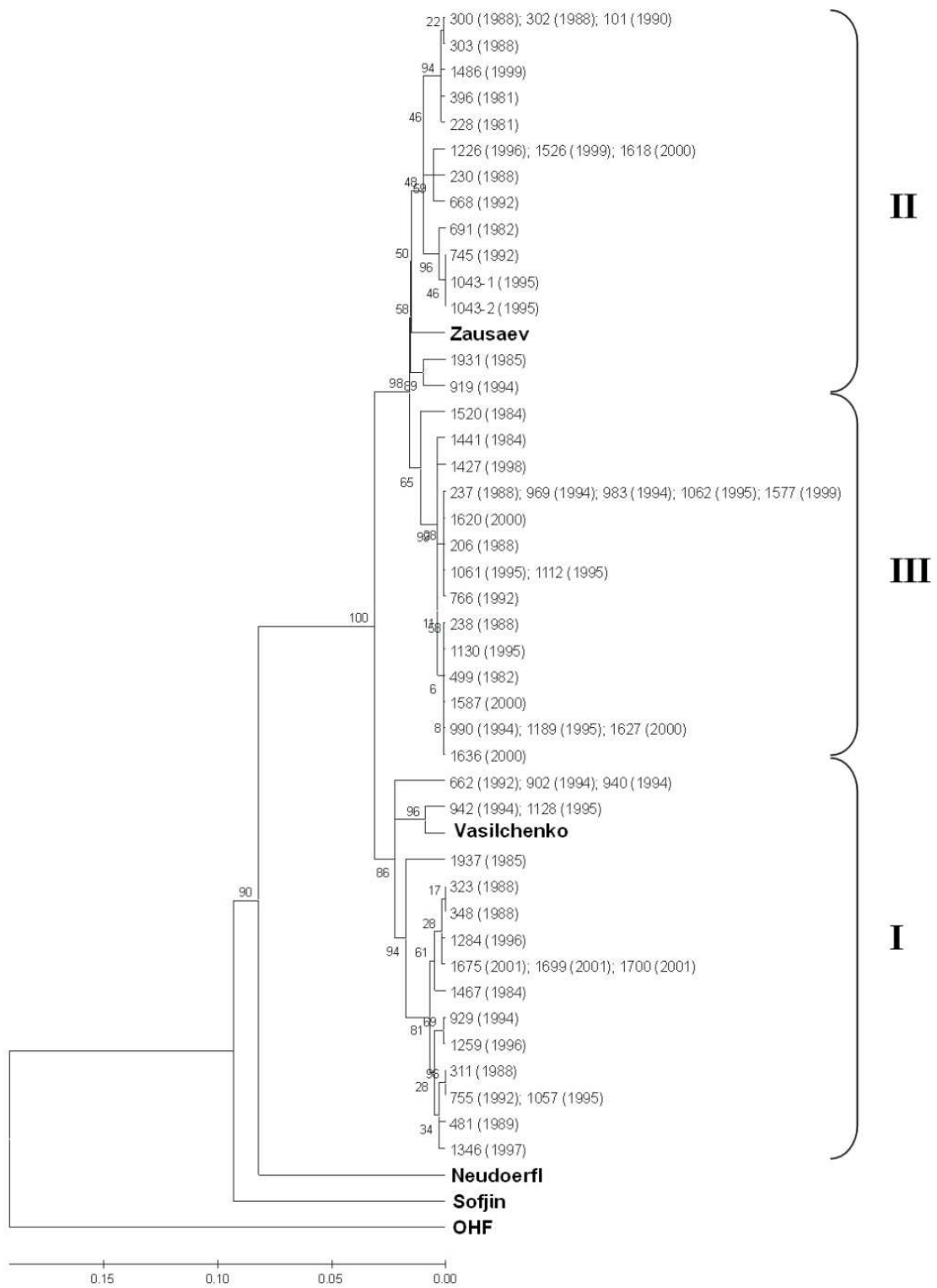


Fig. 5. Dendrogram based on TBEV E gene fragment sequences built by UPGMA method. In brackets - year of strain isolation; Prototype strains marked with bold. I -Vasilchenko subgenotype group; II -Zausaev subgenotype group; III - possible subgenotype non-described earlier

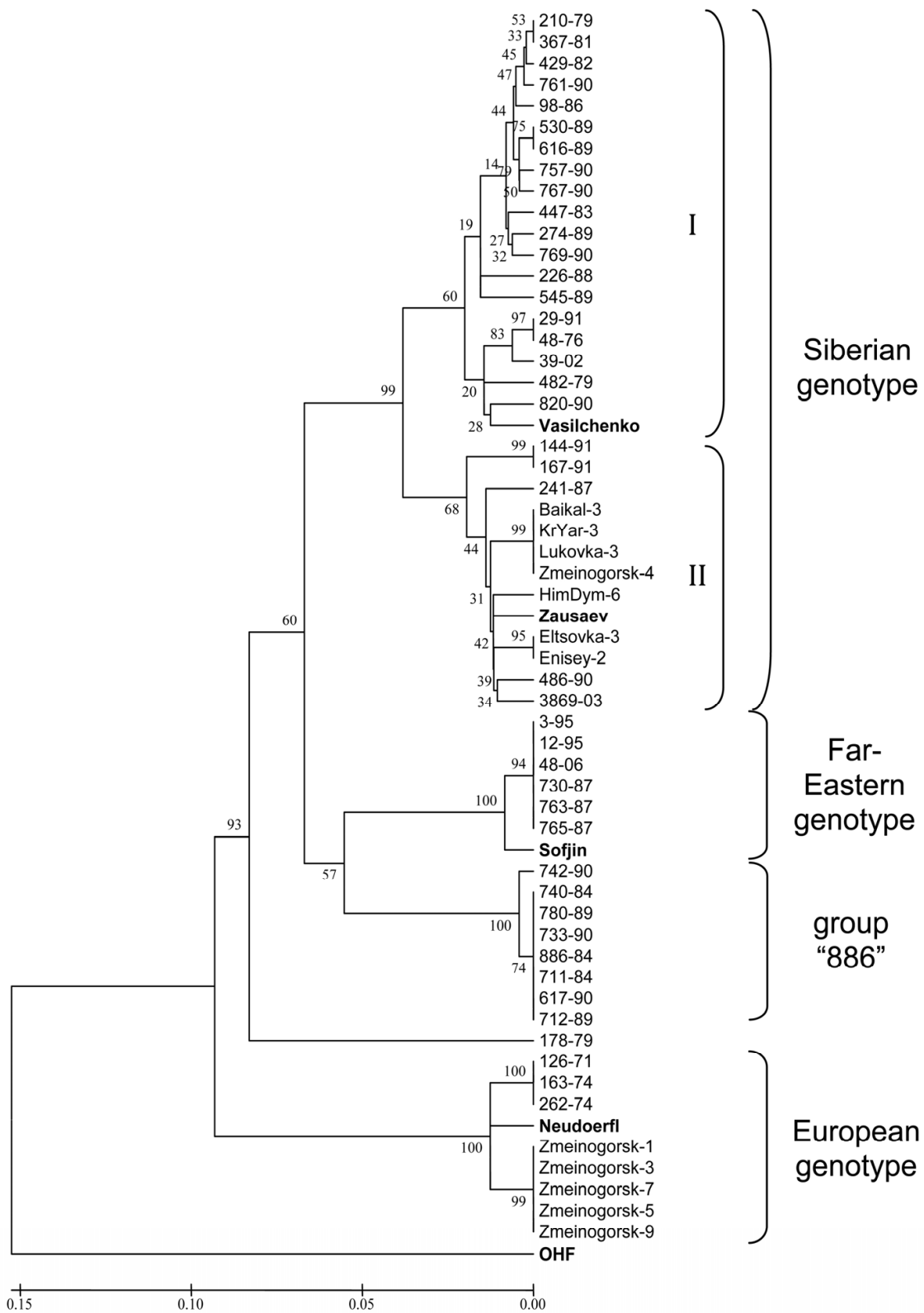


Fig. 6. Dendrogram based on TBEV E-NS1 gene fragment sequences built by UPGMA method. Prototype strains marked with bold; I -Vasilchenko subgenotype group; II -Zausaev subgenotype group

Phylogenetic analysis was performed with Mega 4.0 program [Kumar et al., 2004]. The sequence of Omsk hemorrhagic fever virus genome fragment was used as outgroup. The analysis of dendrogram built by UPGMA method demonstrated that all investigated strains could be divided to three groups: the first one corresponded to Vasilchenko subgenotype TBEV strains (group I), the second one – to Zausaev subgenotype (group II) and for the group III the prototype strain wasn't found (Fig. 5).

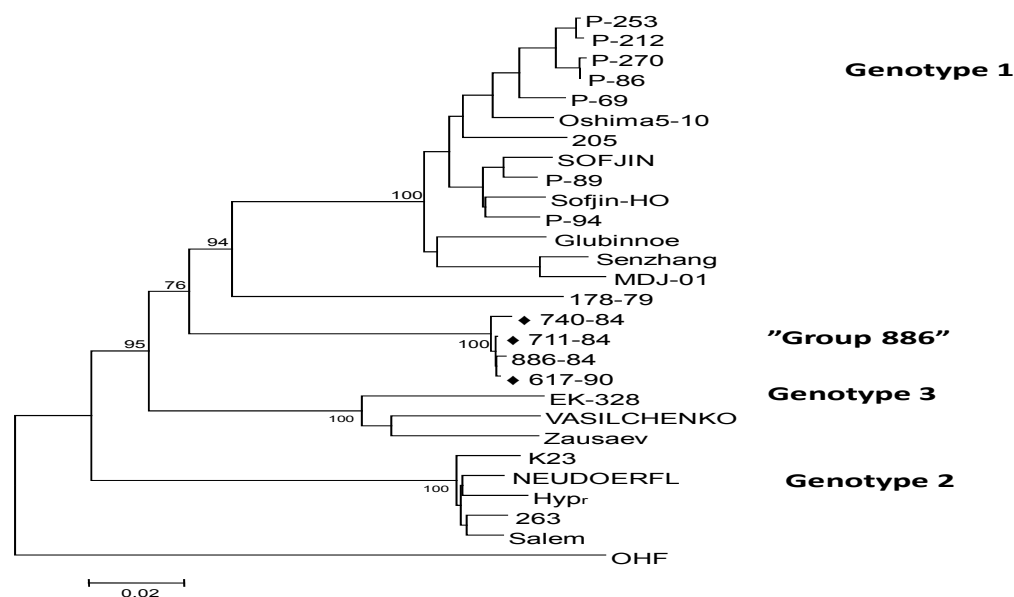
For the phylogenetic analysis of TBEV strains from the collection of Institute of Epidemiology and Microbiology, SCFHHRP ESD RAMS (Irkutsk, Russia) 54 strains from the set used for molecular hybridization of nucleic acids test with genotype-specific deoxyoligonucleotide probes (see part 2 of the Chapter) were chosen. RT-PCR with the primers corresponded to E-NS1 gene fragment was performed followed by sequencing. It was demonstrated that methods of molecular hybridization of nucleic acids with genotype-specific probes and molecular-genetic analysis resulted in identical data. It was shown that TBEV strains from Eastern Siberia could be referred to European (3 strains), Far-Eastern (6 strains) and Siberian (31 strains) genotypes (Fig. 6). The last one was shown to divide only into Vasilchenko and Zausaev subgenotypes. Also five TBEV strains from Altai region (Western Siberia) were found to be European genotype. Also one should pay a special attention to the strain 178-79 and to the group of eight strains including 886-84 strain.

3.5 Sequence analysis of TBEV strains from “group 886” and phylogenetic analysis of strains 178-79 and 886-84

All the strains from “group 886” originated from four natural foci located in the Bichura and Barguzin districts of Buryatia; Krasnochikoisk district of Chita region; and Ekhirit-Bulagatsk district of Irkutsk region. The strains were typed preliminarily as Far-Eastern, albeit with unusual hybridization pattern: 3 of 12 genotype 1 specific probes react with these strains (although all over the surveyed area the strains of genotype 1 with reactivity corresponding probes within the limits of 42–100% circulate). In addition to strain 886-84, this group included strains 740-84 and 711-84 originated from red vole (*Clethrionomys rufocanus*) and seven strains from ticks *I. persulcatus*: 418-90, 712-89, 608-90, 617-90, 636-90, 691-90, and 287-83 isolated in the period from 1983 to 1990.

For strains 617-90, 740-84, and 711-84, the sequences coding protein E (1,322 nt) and NS1 (872 nt) were obtained (EU878283, EU878282, EU878281). Comparison of these sequences with the corresponding genome region of the 886-84 strain (EF469662) revealed a high level of homology (99.3–99.8%) for all four sequences. Differences in the sequences between strains from “group 886” and strains belonging to three genotypes made up 12% (Fig. 7) which corresponds to the criteria of genotyping suggested by Zlobin et al. [2001a, b]. Thus, the genetic evidence of the circulation in Eastern Siberia of genotype 5 strains described earlier by Zlobin and coauthors was obtained.

Phylogenetic analyses confirmed the uniqueness of two East Siberian TBEV strains, 178-79 and 886-84. These strains were located on distinct branches and most often grouped with genotype 1 strains. Depending on the genome region analyzed, these strains adjoined with either genotype 2 or genotype 3 isolates. For example, the phylogenetic trees drawn up for three TBEV genes showed that both East Siberian isolates differed from other 21 TBEV strains, which formed three groups (Fig. 8). Phylogenetic trees drawn up for the other seven TBEV genes possessed the same branching pattern as the bottom tree (“all genome”).



% of sequences (2194 nt) distinction among strains within groups and between groups

	genotype 1	178-79	«group 886»	genotype 2	genotype 3
genotype 1	4,7				
178-79	12,4				
«group 886»	12,8	13,0	0,85		
genotype 2	16,1	16,4	16,3	2,2	
genotype 3	14,8	14,3	13,1	15,6	6,7
OHF	19,9	20,6	19,6	19,1	20,0

Fig. 7. Phylogenetic tree (NJ, Kimura-2-parameter) and differences levels between TBEV strains according to analysis of the genome fragments coding the major part of E and NS1 genes

4. Discussion

Although TBEV strains had gone through several passages in laboratory animals the patterns of RNA hybridization for the strains isolated from the same natural focus for several years were similar. Thus, the results obtained with the panel of probes could be used for characterization of TBEV variants circulating in different natural foci.

The set of investigated in molecular hybridization of nucleic acids test TBEV strains based mostly on the local collection appeared to contain mainly Eastern Siberian variants. The geographic distribution of TBEV genotypes correlated well with the previously published data [Zlobin et al., 2003]. The genotype 2 (European) was found mostly at the territory of Altai (5 of 8 isolates in Western Siberia) (Table I and Fig. 3) and more rarely in Eastern Siberia (5 of 165 isolates). Genotype 1 (Far-Eastern) was found everywhere in minor cases. Genotype 3 (Siberian) was more common in Eastern and Western Siberia.

Four above-mentioned foci located in Eastern Siberia (Bichura and Barguzin districts of Buryatia, Krasnochikoisk district of the Chita region, and Ekhirit-Bulagatsk district of the Irkutsk region) are of special interest due to circulating strains of genotype 5. Notably, within the Ekhirit-Bulagatsk focus, in addition to 886-84 strain, the representatives of all three main genotypes were present but also strains of different subgenotypes and unique

178-79 strain were found. The strain 178-79 was the only one that was recognized by a specific probe E-4. The annealing site for this probe contains several single genotype 2- and genotype 3 specific nucleotide substitutions. From more than 600 TBEV sequences of this region known so far none resembles the sequence of 178-79 strain. The results of this study demonstrated the existence of a relatively stable set of TBEV genetic variants within a specific area, which were recorded for the last 70 years.

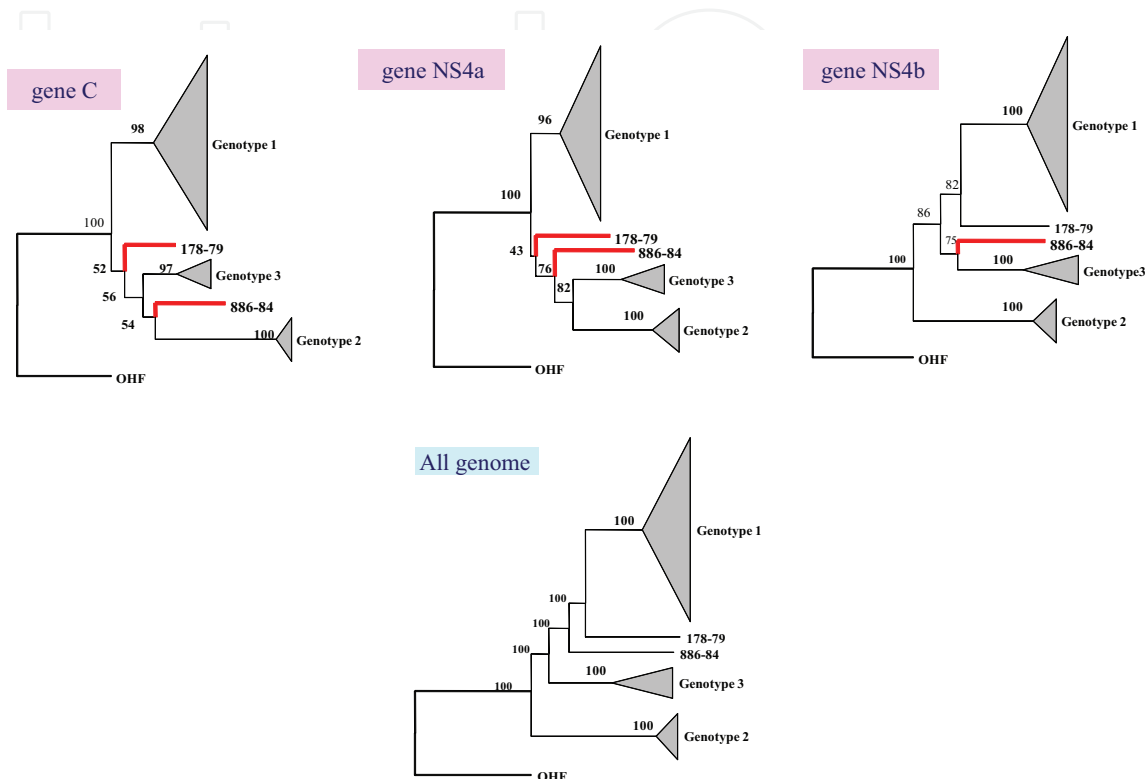


Fig. 8. Phylogenetic tree (NJ, Kimura-2-parameter) and differences levels between TBEV strains according to analysis of the genome fragments coding the major part of E and NS1 genes.

Genotype 1 cluster: Sofjin, Sofin-HO, 205, Oshima5-10, Senzhang, MDJ-01, Glubinnoe, Primorye-69, Primorye-86, Primorye-94, Primorye-212, Primorye-253, Primorye-270 strains.
 Genotype 2 - Neudoerfl, 263, Hypr, K23, Salem strains;
 Genotype 3 - Vasilchenko, Zausaev, Ek-328 strains

These TBEV strains, 178-79 and 886-84 originated from the same natural focus in Eastern Siberia, are of special interest. Their singularity was first observed in the study of serological properties and short E gene sequences [Trukhina et al., 1988; Zlobin et al., 1996, 2001a, b, 2002; Verkhozina et al., 2002]. These studies suggested that the 886-84 strain occupied an intermediate position between the East Siberian and Far Eastern serotypes of TBEV and possessed the properties of both serotypes [Trukhina et al., 1988]. This strain has an identical degree of similarity with all the three subtypes of TBEV in reaction of diffusion precipitation in agar (RDPA) with cross-adsorbed strain-specific sera [Verkhozina et al., 2002; Zlobin et al., 2002]. Pogodina et al. [1981] have included 178-79 strain in the Aina-like strain group (in modern classification – the Ural-Siberian or Siberian genotype). Zlobin et al. [1996] also have come to the conclusion that this strain is clearly differentiated from representatives of other antigen subtypes both in RDPA and in reaction of neutralization. According to the analysis

of E gene fragment sequences (160 nt in length) in 34 TBEV strains the 886-84 and 178-79 strains were not included in any of the known three genotypes, but the analysis of amino acid sequences derived from corresponding nucleotide sequences of the studied fragment has demonstrated that 886-84 strain belongs to genotype 3 and 178-79 strain to genotype 1 [Zlobin et al., 2003]. It should be noted that, according to Zlobin et al. [2001b], genotype 1 corresponded to the FE serotype while genotype 2 corresponded to the Western serotype. Genotype 3 included two antigenic variants, Ural-Siberian and Central Siberian trans-Baikal, or serotype Aina. Corresponding prototype strains for these two variants were Lesopark-11 [Zlobin et al., 1991] and Aina [Pogodina et al., 1981]. Nowadays, both variants belong to the same serotype, Ural-Siberian and Central Siberian trans-Baikal, with the prototype strain Vasilchenko. Uniqueness of isolates 178-79 and 886-84 was shown in the atypical (for genotype 1) hybridization pattern and was also apparent in the phylogenetic trees (Figs. 7 and 8). The existence of this so-called "group 886" showed that TBEV variants of the genotype 5 existed in nature and occupied a certain niche, since they were isolated from different species (rodents and ticks), within different regions in Eastern Siberia, and for several years (1983–1990). These findings raise the question whether the genotype 5 variants are health threatening. Severe TBE cases were observed in the natural foci where TBEV variants from "group 886" circulated [Demina et al., 2007]. These foci are located in Bichura area of Buryatia and Krasnochikoiisk district of Chita region [Lohov and Blinnikova, 1986; Gorin et al., 1992]. Recently, TBEV sequences have been revealed by RT-PCR from a fatal TBE case in Bulgan aymack, Mongolia [Khasnatinov et al., 2009]. This district borders on the four known foci of "group 886" in the south of Eastern Siberia. When compared with the strain 886-84 the 281 nt long fragment of the E gene of this strain showed a high level of sequence homology (98.5%). So it should be important to reveal the origin of these unusual genetic variants of TBEV that remains unknown.

The analysis of existing sequence data of TBEV shows that the arrangement of specific oligonucleotide sequences suitable for genotyping does not always coincide with flashpoints of amino acid mutations and secondly, that genotypic differences are often revealed at the level of the third position in the codon. It means that genotypic differences are possibly connected not only with amino acid substitutions but also with the substitutions in the third positions of codons. So it is possible that natural selection takes place not only at the protein level but also at the RNA level.

5. Conclusions

It was shown that strains of three TBEV genotypes were found at the territories of both Western and Eastern Siberia that was confirmed by both molecular hybridization of nucleic acids test and sequencing followed by phylogenetic analysis. Based on phylogenetic analysis, all strains from Western Siberia corresponded to Siberian genotype were divided into three subgenotypes – Zausaev, Vasilchenko and subgenotype non-described earlier. On the contrary, the strains from Eastern Siberia were divided only into Zausaev and Vasilchenko subgenotypes. Also two groups of strains that could possibly be the new TBEV genotypes meeting the requirements of differences in genome sequences homology level with other genotypes were detected. The fourth possible genotype was presented by only one isolate, 178-79, originated from Irkutsk region, Russia. The fifth possible genotype included 10 isolates, one of them, 886-84, was described earlier. So, the existence of this so-called "group 886" showed that such TBEV variants existed in nature and occupied a certain niche, since they were isolated from different species (rodents and ticks), within different

regions in Eastern Siberia and for several years (1983–1990). It is unknown whether “group 886” variants are health threatening because recently the comparison of genome fragment sequence of TBEV from clinical samples of fatal case in Mongolia revealed a high level of homology (98.5%) with the strain 886-84.

6. Acknowledgements

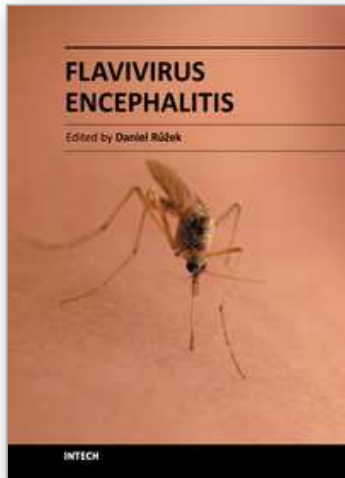
This work was supported in part by interdisciplinary integration projects of SB RAS №6 and 63.

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Flavivirus Encephalitis

Edited by Dr. Daniel Ruzek

ISBN 978-953-307-669-0

Hard cover, 478 pages

Publisher InTech

Published online 30, September, 2011

Published in print edition September, 2011

Encephalitis is an inflammation of the brain tissue associated with clinical evidence of brain dysfunction. The disease is of high public health importance worldwide due to its high morbidity and mortality. Flaviviruses, such as tick-borne encephalitis virus, Japanese encephalitis virus, Murray Valley encephalitis virus, or St. Louis encephalitis virus, represent important causative agents of encephalitis in humans in various parts of the world. The book *Flavivirus Encephalitis* provides the most recent information about selected aspects associated with encephalitic flaviviruses. The book contains chapters that cover a wide spectrum of subjects including flavivirus biology, virus-host interactions, role of vectors in disease epidemiology, neurological dengue, and West Nile encephalitis. Special attention is paid to tick-borne encephalitis and Japanese encephalitis viruses. The book uniquely combines up-to-date reviews with cutting-edge original research data, and provides a condensed source of information for clinicians, virologists, pathologists, immunologists, as well as for students of medicine or life sciences.

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