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Tick-Borne Encephalitis Virus Quasispecies Rearrangements in Ticks and Mammals

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1. Introduction

Tick-borne encephalitis (TBE) is one of the most dangerous flavivirus infection of the central nervous system in Eurasia. The etiological agent, the TBE virus (TBEV) is transmitted to man by tick bytes. Geographic natural habitat of the TBEV appeared to be discontinuous and extended throughout southern part of Eurasian forest belt from Pacific to Atlantic ocean mainly within distribution areas of the virus vectors – ixodid ticks. TBE outbreaks have been registered in northern China and Japan in the east, through Russia to about 30 European countries including France, northern Italy and Norway in the west with the highest morbidity rates in Russia, Austria, Czech Republic, Slovenia and the Baltics (Latvia, Lithuania and Estonia) (Gritsun et al, 2003; Mansfield et al., 2009). In France, Italy, Greece, Norway and Denmark, TBE is of minor importance. In the United Kingdom, Ireland, Belgium, the Netherlands, Luxembourg, Spain and Portugal, TBE is not indigenous and no TBE cases have been reported yet. Detailed epidemiological statistics from 1990 onwards can be obtained from the website of the International Scientific Working Group on TBE [http://www.isw-tbe.info]. The TBE prevalence is increasing worldwide with spread of the virus to previously non-endemic countries in Europe (France) and Asia (China, Mongolia, Japan and South Korea). TBEV is listed as a category C agent on the Centers for Disease Control and Prevention (USA) list of select biological agents.

The TBEV persists in endemic regions or so-called natural foci, where it circulates among vertebrate hosts (mainly small rodents and insectivorous) and the arthropod vectors (ticks). Years of observations of the distribution of TBE incidences suggest that such natural foci are very stable. Formation, development and stability of the TBE natural foci are determined by the coincidence of several ecological factors including temperature, relative air humidity, soil humidity, biotope vegetation, population density and dynamics of ixodid ticks and their hosts, susceptibility of reservoir hosts to the TBEV, proportion of immune hosts as well as the virus prevalence among both ticks and vertebrate hosts (Hofmann, 1973; Plassmann, 1980; L'vov et al., 1989; Korotkov et al., 2007; Tick-Borne Encephalitis (TBE) and its Immunoprophylaxis, 1996 and references therein). Reservoir host species abundance, multiple transmission cycles and adaptation of the TBEV to different hosts cause long-term resistance of the parasitic system in endemic regions.

The TBEV as a typical member of the *Flaviviridae* family possesses a single-stranded, positive genomic RNA of approximately 11 kb in length, containing a 5′ cap and lacking a 3′-terminal polyadenylate tail (Gritsun et al., 2003; Mansfield et al., 2009 and references therein). Molecular typing of the TBEV using different methods (ELISA, molecular hybridization with radioactive oligonucleotide probes and RT-PCR with subsequent sequencing of E gene) revealed 3 main subtypes: Far Eastern, Siberian and European (Pogodina et al., 1981; Zlobin et al., 1996; Ecker et al., 1999; Bakhvalova et al., 2000).

Currently, according to the International Committee for Taxonomy of Viruses, TBEV is classified as one species with three subtypes, namely the Far Eastern subtype (mainly isolates from far-eastern Russia, China and Japan), currently widely spread Siberian subtype (earlier isolates from Eastern and Western Siberia, Urals and far-eastern Russia and at present dominant subtype in many TBE endemic regions of Russia and surrounding countries, gradually replacing 2 other TBEV subtype (Pogodina et al., 2007) and the European subtype (which comprises almost all known isolates from Europe) (Gritsun et al., 2003; Mantke et al., 2008; Mansfield et al., 2009 and references therein). The 3 TBEV subtypes are associated with varying degrees of disease severity. Human infections with Far Eastern subtype viruses are usually severe, frequently with encephalitic symptoms (focal meningoencephalitis or polyencephalitis), with an associated fatality rate between 5 and 35% (earlier 20-60%). This type does not cause chronic disease. In contrast, infection with the TBEV Siberian subtype cause a less severe disease (fatality rate between 1 and 3% (earlier 6-8%)), with a tendency for patients to develop chronic or extremely prolonged infections accompanied by diverse neurological and/or neuropsychiatric symptoms. In contrast to these two forms, infections caused by European strains typically take a biphasic course: the first (viraemic) phase presents as an influenza-like illness lasting 1–8 days with fever, malaise, headache, myalgia, gastrointestinal symptoms, leukocytopenia, thrombocytopenia and elevated liver enzymes, often followed by a symptom-free interval of about 1–33 days and the second phase in 20–30% of infected patients with clinical features of different severity (meningitis, meningoencephalitis, meningoencephalomyelitis or meningoencephaloradiculitis), the appearance of specific antibodies in the serum and cerebrospinal fluid and the fatality rate less than 2% (Gritsun et al., 2003; Mantke et al., 2008; Mansfield et al., 2009 and references therein). Accordingly, ratio of different TBEV subtypes could influence on the TBE severity.

Recently, a new classification of the tick-borne flaviviruses based on phylogenetic analysis of complete coding sequences of their genomes had been proposed (Grard et al., 2007). According to the recommendation the tick-borne flaviviruses should be divided into 4 types (i.e. Western tick-borne encephalitis virus, Eastern tick-borne encephalitis virus, Turkish sheep tick-borne encephalitis virus and louping ill tick-borne encephalitis virus) (Grard et al., 2007). However, it has not yet been accepted by the International Committee on Taxonomy of Viruses.

Moreover, new phylogenetically distant variants of the TBEV isolated in Irkutsk endemic region, Eastern Siberia, Russia and Mongolia that do not belong to any known genetic subtype (Khasnatinov et al., 2010; GenBank accession numbers EF469662 and EF469661 for strains 886-84 and 178-79, respectively).

Ticks are known to be main carriers (so-called vectors) and reservoir hosts of the TBEV in nature. Their ability to feed on a variety of vertebrate animals, intracellular digestion of blood and their long life cycle for 3-6 years and feeding at each stage of development make them ideal vectors for many tick-borne infectious agents including the TBEV. Natural TBEV

infection had been revealed for 16 species of ixodid ticks (Korenberg, 1989). Thus, in Central Europe TBEV was revealed in 8 species of "hard" ticks having a dorsal shield: *Ixodes persulcatus* Schulze, *Ixodes ricinus* L., *Ixodes hexagonus, Ixodes arboricola, Haemaphysalis punctata, Haemaphysalis concinna* Koch, *Dermacentor marginatus* Sulz. and *Dermacentor reticulatus* (Tick-Borne Encephalitis and references therein). In the Western Siberia population of ixodid ticks in the TBE endemic regions included *I. persulcatus* Sch., *I. ricinus* L., *Ixodes pavlovskyi* Pom., *D. reticulatus* Fabr., *D. marginatus* Sulz., *Dermacentor silvarum* Ol., *Dermacentor nuttalli* Ol., *H.concinna* Koch (Bogdanov, 2006).

The distribution of TBE may be determined by the occurrence of the respective tick vectors in certain regions. While *Ixodes ricinus* is the prevalent hard tick species across Europe and therefore the most important transmitter of the TBEV European subtype, *Ixodes persulcatus* inhabits in forest regions of the Urals, Siberia, far-eastern Russia and China and is the main vector of the other subtypes. Co-circulation of two or all three subtypes could be shown for Finland and the Baltic states where the distribution areas of the two main tick species overlap. The TBEV can be transmitted to vertebrate hosts or man by larvae, nymphs or adult ticks. Ticks provide the TBEV persistence during each stage of development, the virus amplification as well as both transovarial and transstadial transfer (Balashov, 2010). The TBEV was described to activate ticks in their host pursuit and to induce hormone of molt thus reducing a period of a tick development (Alekseev, 1990; Korotkov, Burencova, 2006).

The ixodid ticks parasitize more than 100 different species of mammals, birds, reptiles and amphibians thus providing the TBEV transmission and the vertebrate reservoir hosts involvement into epizootic process (Levkovich et al., 1967; Filippova, 1985). For most vertebrate natural hosts, TBEV is apathogenic without any infection manifestations but induces the virus-specific antibodies (Bakhvalova et al., 2006 and references therein). The TBEV is capable for lifelong persistence with reduced reproduction activity mainly within mammal cells to escape from extracellular specific antibodies (Bakhvalova et al., 2006 and references therein).

Transmission of the TBEV in the wild takes place in different interrelated ways. During feeding of ticks on animals, either viremic or nonviremic transmission can take place (Bakhvalova et al., 2006 and references therein). In addition, vertical transovarial, transstadial and horizontal sexual transmission may take place between both ticks and warm-blooded hosts (Bakhvalova et al., 2009 and references therein). TBEV maintenance mechanisms may differ in acute and persistent infection (Bakhvalova et al., 2006 and references therein).

Evolution of flaviviruses evidently depends on their hosts (Zanotto et al.1995, Gould et al. 1997). Arthtopod-borne viruses reproduce in distantly related hosts such as vertebrates and invertebrates. Essential differences of host organisms cause selection of certain genetic variants of flavivirus quasispecies. Tick-adapted variants of TBEV were shown to exhibit small-plaque phenotype and slower replication in mammal cells, decreased neuroinvasiveness in laboratory mice and higher yield in laboratory line of ticks caused by 15 nucleotide substitutions (Romanova et al., 2007). Mammal-adapted variants possess inherent inverse properties. Host switch induce changes in ratio of the host-adapted variants. Tick-adapted variants might have selective advantages during reproduction of the virus in ticks during diapause without involving mammal hosts. Similarly, during TBEV persistence in mammals with vertical transmission from parents to their progeny, the mammal-adapted variants could accumulate and prevail. However, the TBEV adaptation to different hosts in the wild under natural conditions remains unclear.

Our aim was to compare the TBEV infection of ticks and small mammals in an endemic region for 30 years of observation.

2. Ticks and tick-adapted variants of the TBEV

Study of the TBE parasitic system includes system analysis of dynamics and interrelationships of all 3 components: TBEV with detection, identification and viral load quantitation, population density and dynamics of both ticks and vertebrate hosts.

TBE epidemiology is closely related to the ecology and biology of ticks. Throughout the world about 850 tick species are classified into 2 families: The *Ixodidae* (ixodid or hard ticks) and the *Argasidae* (argasid or soft ticks). Ticks transmit viruses, bacteria, fungi, protozoa and nematodes. Approximately 25% of the viruses isolated from field-collected arthropods are from ticks. At least 14 from more than 70 known flaviviruses are transmitted by ticks, generally ixodid species. Digestive process is an unusual feature of ticks from other arthropods. Except for hemolysis of the blood cells in the midgut lumen, digestion in ticks is entirely intracellular, a process known as heterophagy. All digestion is accomplished within epithelial cells of midgut. Tick saliva apparently lacks hemolytic enzymes, and the erythrocytes and other cellular elements are ingested unchanged. Blood taken into the midgut of the tick remains largely undigested for long periods, therefore viruses and bacteria remain in nonhostile environment, favors virus entry as extracellular virions and via infected host cells. The undigested blood meal remains as a food reserve. Therefore, ticks are able to survive for extended periods of starvation (Nutall and Labuda, 2003). Ixodid ticks feed for periods ranging from a few days to several weeks. To concentrate the dilute blood meal, ticks use their salivary glands to periodically secrete excess water from the blood meal back into the host. All developmental stages (except males) consume many times their original body weight in blood and other fluid, mated females often imbibe more than 100 times their unfed body weight. The ability of ticks to produce thousands of eggs is important in their population dynamics. An *I. ricinus* or *I. persulcatus* females are capable of laying 350 to 5000 eggs, depending mostly on the amount of consumed blood. Immature stages of *I. ricinus* and *I. persulcatus* often feed on rodents and birds, whereas adults feed on goats and other ungulates (Nutall and Labuda, 2003). In nature, once infected ticks at each developmental stage remain permanently infected.

Our research was carried out in a recreation zone of Novosibirsk (54°49′ N, 83°05′ E), South-Western Siberia, Russia. For the area temperature is optimal but humidity is rather limited factor for ixodid ticks. Therefore, population density of ticks in Western Siberia is relatively low with high TBEV infection rate (Fig. 1). Since 1980 population density of the ixodid ticks varied from 4.4 to 51.8 ticks/km. One should note that earlier in the region *I. persulcatus* absolutely prevailed, however during the last years rate of *I. pavlovskyi Pom.* in urban and suburban biotopes was significantly grown up to 80% from total ixodid tick population in 2011. Whereas in distant forest areas located at 10-15 km from the city the ratio of *I. pavlovskyi* did not exceed 15%. Similar observations were previously described for other Western Siberian region [Romanenko and Kondratieva, 2010). The changes in ixodid tick fauna might be caused by anthropogenic pressure.

Until now, surveillance of the TBEV infection rate in different endemic regions of Eurasia is not uniform and may affect the prevalence estimates. Thus, currently available data from different countries are difficult to compare. Furthermore, little is known about the true TBEV prevalence in tick populations or about the circulation of new genetic subtypes and hemagglutination-deficient variants. TBEV prevalence in the tick population of endemic areas in Austria was revealed by bioassays to be 0.44-6.2%, in Czech Republic – 0.30-4.50%, in Finland – 0.07-2.56%, in Germany – 0.2-2.0%, in Italy – 0.05%, in Sweden 0.1-1.0%, in Switzerland – 0.1-1.36 and in Russian Federation 3.0-40.0% (Tick-Borne Encephalitis (TBE)

and its Immunoprophylaxis, 1996 and references therein). Since discovery of the TBEV in 1937 various methods such as bioassays with infection of permissive tissue cultures or laboratory suckling mice, ELISA, direct and reverse molecular hybridization of nucleic acids (MHNA), reverse transcription (RT) with subsequent PCR and detection of their products by electrophoresis or by fluorescence measurements in real time were used for the virus detection. Currently, the European Network for Diagnostics of "Imported" Viral Diseases (ENIVD) suggests to improve the diagnostics and monitoring of encephalitis viruses in Europe (Mantke et al., 2008).

In Russia tick suspensions from pool (10 ticks in each one) were used for infection of newborn or 2-week-old mice with subsequent identification of the TBEV isolates in serological reactions. TBEV infection rate of adult ticks collected from vegetation varied from 3 to 31.1% of pools of ticks (or from 0.3 to 3.7% of individual ticks) (Beklemishev, 1963) (Fig. 1). Long-term dynamics of population density and TBEV prevalence in ticks were earlier supposed to have positive correlation (Neckiy, Bogdanov,1966). However, later on positive correlation between an abundance and TBEV infection rate of ticks was indeed registered only in natural foci with optimal conditions for tick development, particularly in Far East of Russia (Khabarovsk region) (Vereta, 1975; Vershinskyi, 1984; Okulova, 1986). For taiga endemic regions of Western Siberia correlation was tended to be rather negative (Katin and Pustovalov, 1983). Natural conditions of forest-steppe near Ob river are unfavourable for *I. persulcatus* (Dobrotvorskyi et al., 1994) with diapausa stage for essential part of nymphs. The correlation coefficient between the abundance and TBEV prevalence in ticks appeared to be r = - 0,45, p = 0,012 during period 1980-2010. The negative correlation (r = - 0,75) is especially evident during the last 6 years (Fig.1).

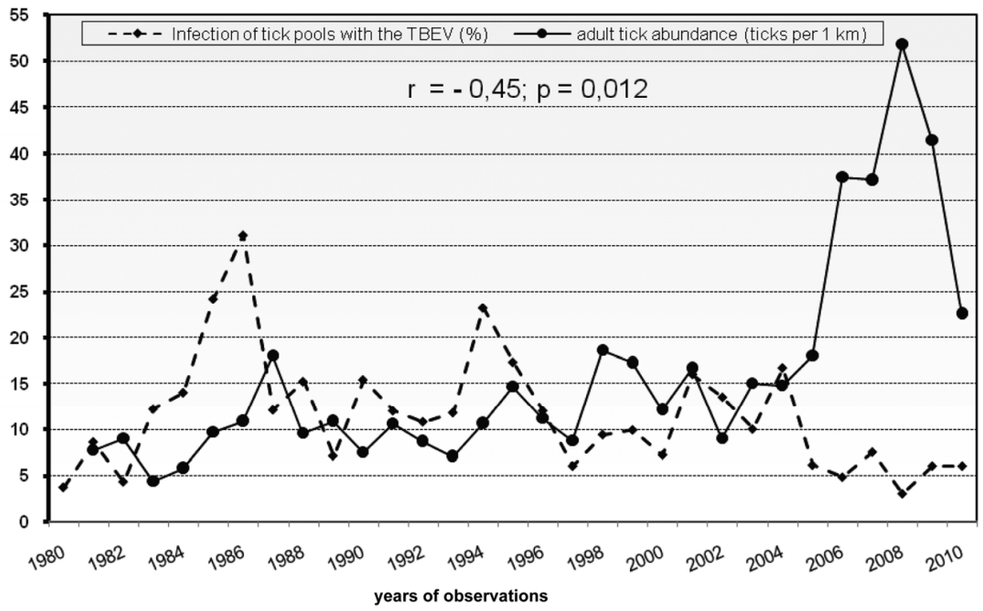


Fig. 1. Long-term dynamics of ixodid ticks abundance and the TBEV infection prevalence

TBEV is typically a hemagglutinating (HA) virus. Ability to agglutinate goose erythrocytes reflects the binding of the TBEV with host cell receptors with subsequent fusion activity and the virus penetration into cells. Whilst most strains of TBEV show HA activity, during the

past 10 years atypical HA-deficient strains have been isolated with increasing frequencies in Europe from both ticks and patients. More than 40 HA-deficient strains are now recognized and all of them exhibit reduced pathogenicity for mice in comparison with HA-competent strains (Khasnatinov et al., 2009). The isolation of HA-lacking strains of TBEV from wild ticks from natural populations and from patients confirmed earlier described tick-adapted laboratory TBEV variants with decreased neuroinvasiveness in mice and absence of HA activity (Romanova et al., 2007). However, for laboratory tick-adapted variant of the TBEV Siberian strain EK-328 only 2 amino acid substitutions in the virion surface glycoprotein E - E122G and T426I were found (Romanova et al., 2007), whereas for wild TBEV HA-deficient isolates of European subtype – 3 mutations D67G, E122G and D277A in the envelope protein (Khasnatinov et al., 2009), with the only change E122G being common for all HA-deficient tick-adapted variants. Multiple alignment of amino acid sequences of the TBEV E glycoprotein revealed that mutation E122G is rare event and was not found in our collection of 255 TBEV strains isolated from ticks in Novosibirsk region, South-Western Siberia, Russia during 1980-2010. The one thing that is evident is the constancy of E122. If that amino acid is associated with the mammalian phenotype as described by (Romanova et al., 2007), then a single passage of the tick-adapted strain in mammalian cells may be sufficient to result in the emergence of the mammalian phenotype in most of the quasispecies.

For other tick-borne flavivirus Powassan despite quasispecies rearrangements with average frequency 0.0123% mutations/nucleotide all sequences derived directly from ticks without a passage in mammalian cells included E122 and V427 (427 corresponds to 426 in TBEV) (Ebel, 2009) as previously suggested to be associated with mammal-adapted variants (Romanova et al., 2007). Therefore, the host-induced alternations of amino acids at those positions could hardly cause tick- or mammalian cell-adapted TBEV phenotypes.

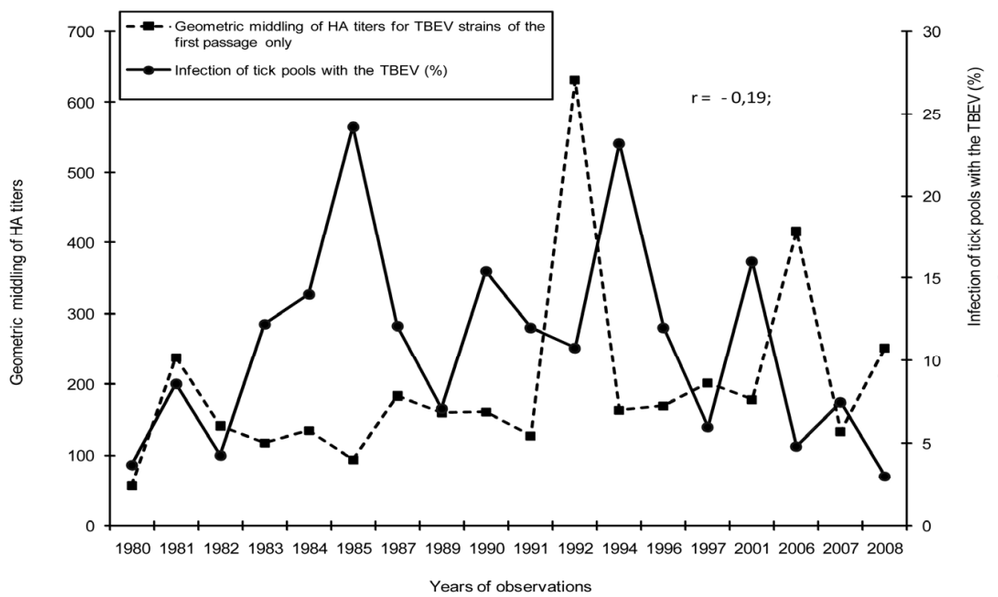


Fig. 2. Reverse correlation between the TBEV prevalence in ticks and hemagglutination (HA) of the TBEV strains for 1980-2008.

Note: geometric middling of HA titers for TBEV strains of the first passage only. For missing years HA titers were determined for other passages of the virus strains

Comparison of long-term dynamics of TBEV prevalence in ticks and HA of the virus strains isolated from the ticks demonstrated slightly negative correlation (r = - 0,19) (Fig. 2). Growth of TBEV-infected tick proportion might cause selection of tick-adapted variants with decreased HA (Romanova et al., 2007; Khasnatinov et al., 2009).

Since 2006 TBEV detection in ticks was performed by using independent methods of bioassays with laboratory white suckling mice, ELISA for the TBEV antigen detection E, reverse transcription with subsequent real-time PCR with primers and fluorescent hydrolysis probes corresponding to the TBEV NS1 or NS5 gene of Siberian or Far Eastern genetic subtype or nested PCR with primer pairs specific to gene E (Bakhvalova et al., 2006; 2009). Molecular methods always confirmed bioassay data but annually allowed us to reveal additional positive samples among tick pools containing apathogenic HA-lacking TBEV or its components – viral RNA and/or glycoprotein E (Fig. 3). Additional passages of the RT-PCR and/or ELISA positive samples using 2-3-days-old mice did not result in TBE symptoms and HA activity but accumulation of TBEV RNA and protein E was observed. The most statistically significant increase of ELISA- and/or RT-PCR-positive samples ($p<0,001$) was found in 2010 (Fig. 3) compared to steady pathogenic virus prevalence.

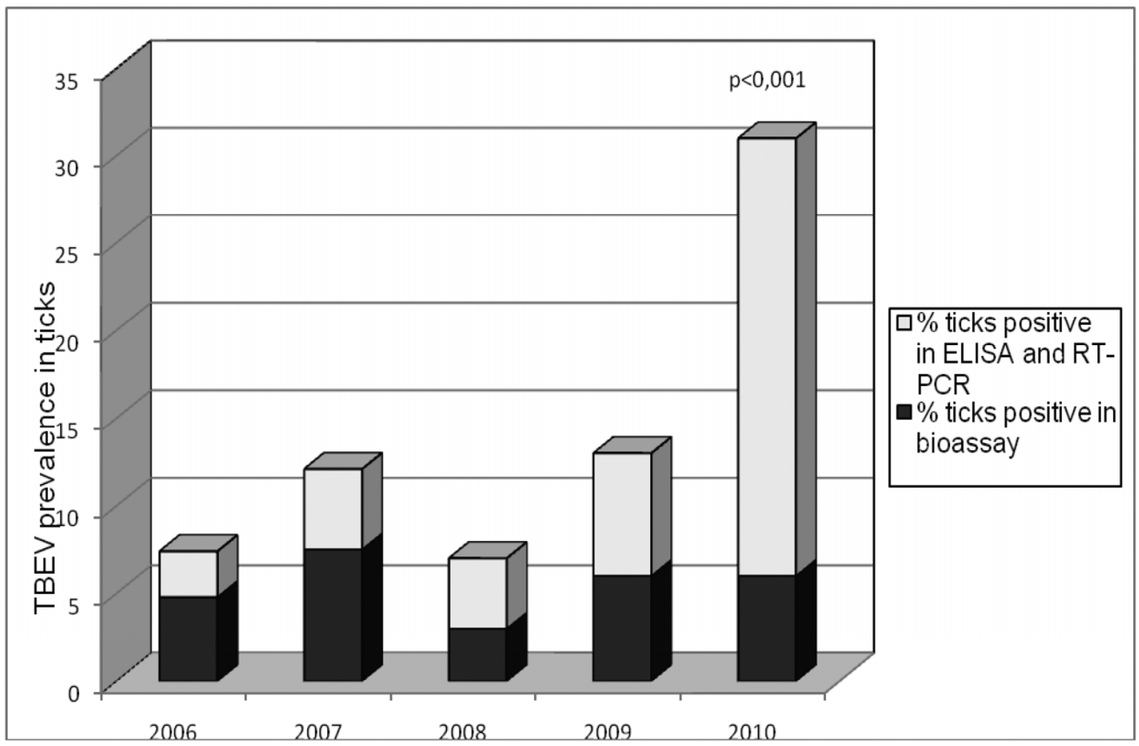


Fig. 3. TBEV detection in pools of unfed adult ticks from Novosibirsk endemic region, South-Western Siberia, Russia by using bioassays (black) and molecular approaches (grey)

During recent decades the epidemiology of the TBEV appears to have been changing, with Siberian genetic subtype gradually replacing Far Eastern and Western European subtypes (Pogodina et al., 2007; Khasnatinov et al., 2009 and references therein). Moreover, Siberian subtype is being isolated more frequently from patients with severe encephalitis. All the TBEV strains isolated for 1980-2010 from ticks in Novosibirsk region, South-Western Siberia,

Russia appeared to belong to Siberian genetic subtype (Morozova et al., 2007). However, real-time RT-PCR with subtype-specific fluorescent probes revealed both Far Eastern and Siberian subtypes with partly mixed infection directly in tick suspensions (Fig. 4).
Quantitation of TBEV RNA using reverse transcription with subsequent real-time PCR with fluorescent hydrolysis probes showed a variation of threshold cycles (Ct) within a range 21-33 for cDNA derived from infected ticks (Fig. 5). According to our calibration with purified plasmid DNA containing cloned full-length DNA copy of the TBEV RNA and taking into account the reverse transcription efficiency as 50% these Ct corresponded to 10^2 -10^7 RNA genome-equivalents per each tick. Analysis of chronological rows of TBEV strains can be performed after numerous passages in mice or tissue cultures with possible quasispecies rearrangements in various hosts.

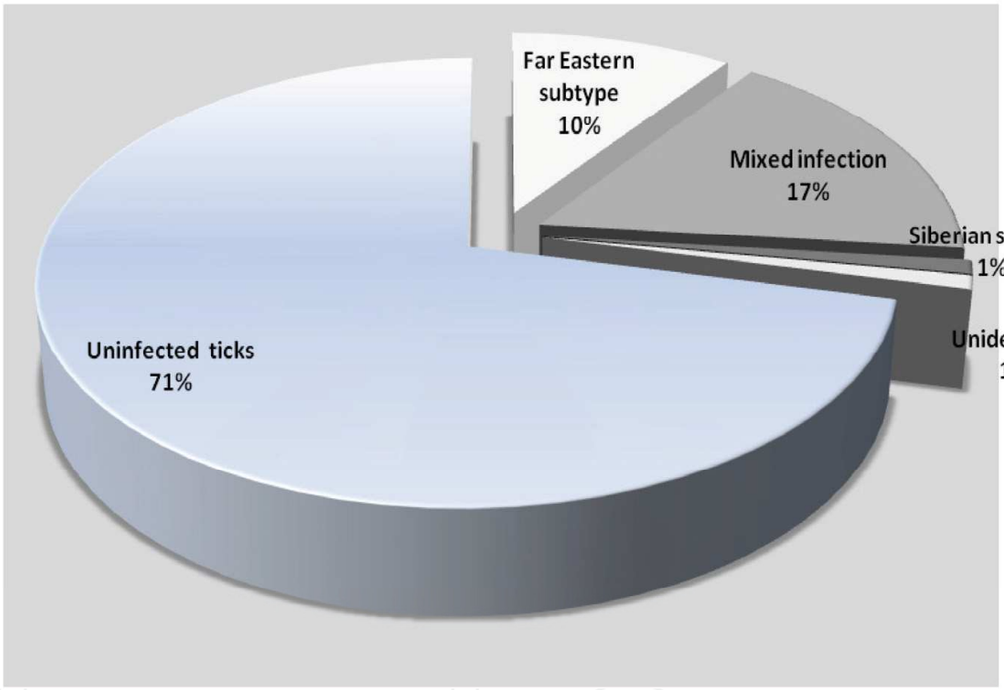


Fig. 4. Ratio of different TBEV subtypes in tick suspensions in 2010

3. Vertebrate reservoir hosts of the TBEV and mammal-adapted variants of the virus

Ixodid ticks feed on more than 100 species of vertebrate hosts including mammals, reptiles, amphibians and birds (Filippova, 1985). Coevolution of viruses with their hosts toward less deleterious infections ensures the survival of both host and virus. Therefore, most wild vertebrates are susceptible and resistant to both mosquito- and tick-borne flaviviruses. Resistance to flavivirus-induced diseases in wild mammals is conferred by the autosomal *Flv*-resistance gene recently identified as 2'-5' oligoadenylate synthetase 1b (*oas*1b) gene that expresses constitutively, does not require interferon induction and inhibits flavivirus replication (Brinton and Perelygin, 2003). Resistant animals are infected productively by flaviviruses but produce lower virus titers, especially in their brains.

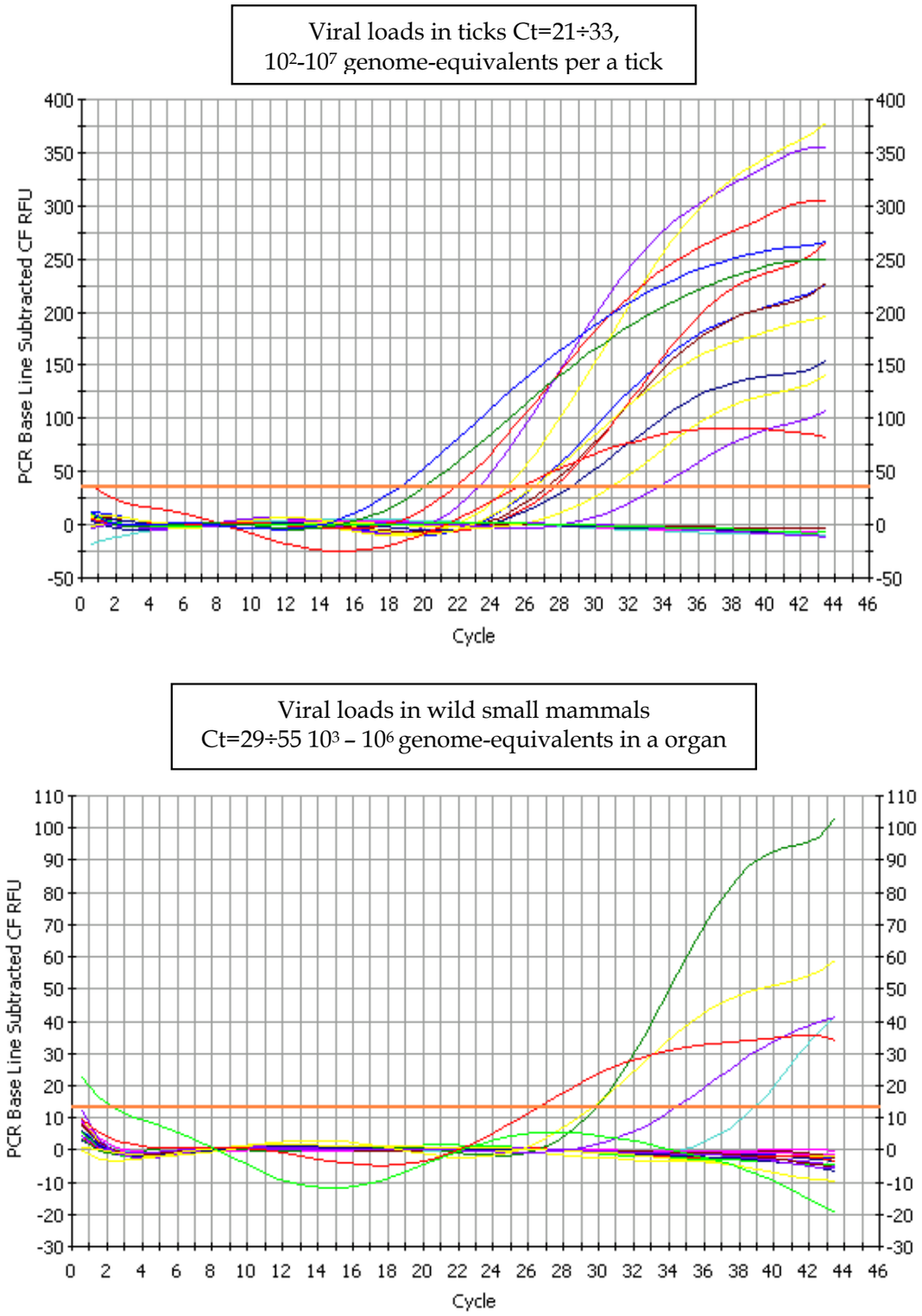


Fig. 5. Real-time PCR quantitation of the viral loads in ticks and small mammals from Novosibirsk endemic region, Western Siberia, Russia

Small mammals mainly rodents (*Rodentia)* and insectivorouses (*Insectivora*) with high reproduction rates and their life span even shorter than that of ixodid ticks are natural hosts for both immature ticks (mainly larvaes) and TBEV. Several small rodent species are highly competent in supporting the tick-borne transmission of TBEV, whereas avian species are not. Regardless the sensitivity of vertebrate hosts to the TBEV their susceptibility estimated by viraemia after infection essentially differs (Levkovich et al., 1967; Chunikhin et al., 1990). A long viraemic stage (2-8 days) along with a high virus titer was observed in small mammals such as field mice, red voles, common voles etc. The duration of viraemia is influenced by the environmental and body temperature with prolongation up to 8-14 days in cold seasons. In large mammals (roes, goats) viraemia is short-lived and only low virus titers were revealed. During the viraemic stage, milk from goats, cows and sheep contains the virus and may serve as the infection source for man. Birds only pass through a very short viraemic stage and play no role as reservoirs of TBEV. But often they serve as hosts for the immature stage of ticks and thus may contribute to the spread of infected ticks (Tick-Borne Encephalitis (TBE) and its Immunoprophylaxis, 1996 and references therein). Regardless of species variations and regional features life-long persistence of apathogenic TBEV in the presence of the virus-specific antibodies is typical for small rodents and insectivorous (Bakhvalova et al., 2006 and references therein).

Conventional estimations of the TBEV prevalence in small mammals are based on both virological and serological data with currently evident limitations. Despite the presence of specific antibodies serves as an evidence of TBEV infection in animals seronegative data may be consequences of immunocompromised or even immunodeficient individuals but not necessarily prove the absence of contacts with the virus. Isolation of the TBEV strains from wild adapted mammals is hardly possible because of the TBEV trace amounts and unpredictable viraemic period since the infection time remains uknown. Molecular approaches including RT-PCR permit to reveal a real infection rate. In our research both serological (ELISA and hemagglutination inhibition (HI) test), RT-nested PCR with subsequent sequencing and real-time PCR with fluorescent hydrolysis probes were used. In the endemic region studied wild small mammals include 9 species of insectivorous with dominant species of common shrew *(Sorex araneus* L.*)* and 12 species of wild small rodents excluding synanthropic rodent species associated with human societies. Rearrangements of dominance structure among rodents are unessential and total population remains stable (Litvinov et al., 2010). Analysis of long-term population dynamics of small rodents and insectivorous in the natural focus of Novosibirsk region, South-Western Siberia, Russia showed their similarity $r = 0,56$; $p = 0,001$ (Fig. 6), that might be explained by similar habitation abiotic and biotic (such as carnivoreses and epizootic infections) factors.

During last 30 years (1980-2010) population densities of small mammals periodically changed from 7.5 to 108.7 animals per 100 cylinder-days. On the average, among trapped small mammals 36% were rodents, 64% - insectivorous. In spite of relative minority, small rodents fed for approximately 90% of immature ixodid ticks, among them 45%-77% were found on red voles *Myodes rutilus* Pallas (earlier *Clethrionomys rutilus* Pallas) and field mice *Apodemus agrarius* Pallas. In many seasons of tick activity in Siberia lasting from May to August relative abundance of immature ticks was maximal for red voles with mean abundance up to 11.2 ticks per 1 animal (Bakhvalova et al., 2006).

TBEV-specific HI antibodies were also found mainly in small rodents and especially in red voles. For many years HI antibodies were not detected in insectivorous sera at all. For the last 4 years levels of seropositive small mammals were high with relative frequencies for red voles – up to 61.9±5.4% and for common shrews - 14.3±7.8%.

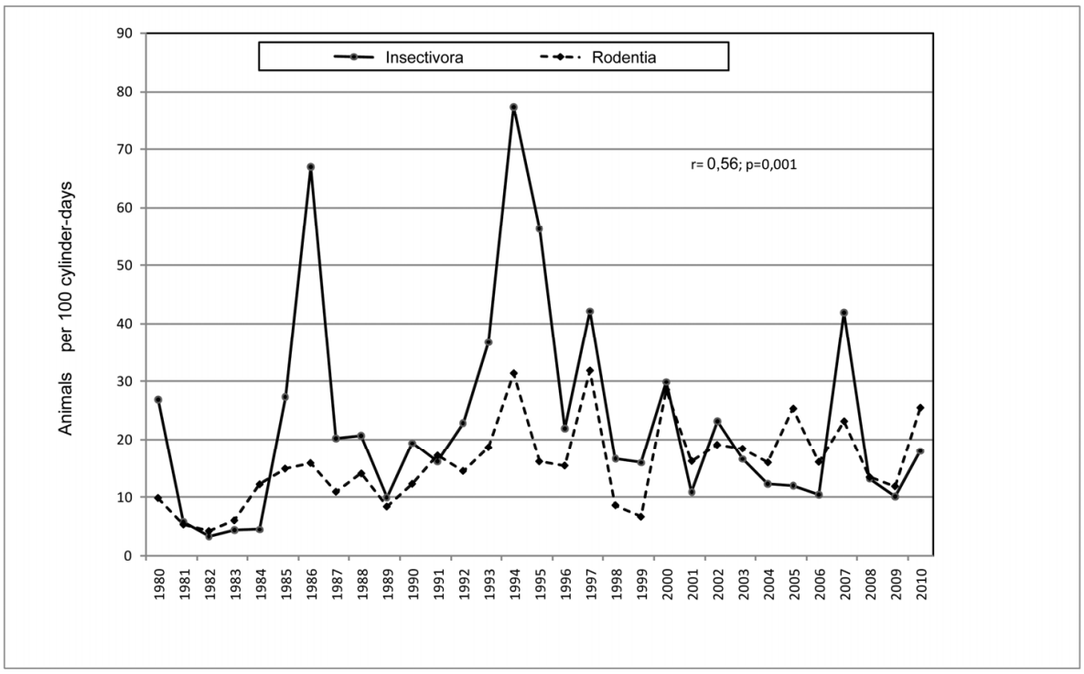


Fig. 6. Long-term population dynamics of small mammals of *Rodentia* and *Insectivora* in recreation zone of Novosibirsk endemic region, South-Western Siberia, Russia

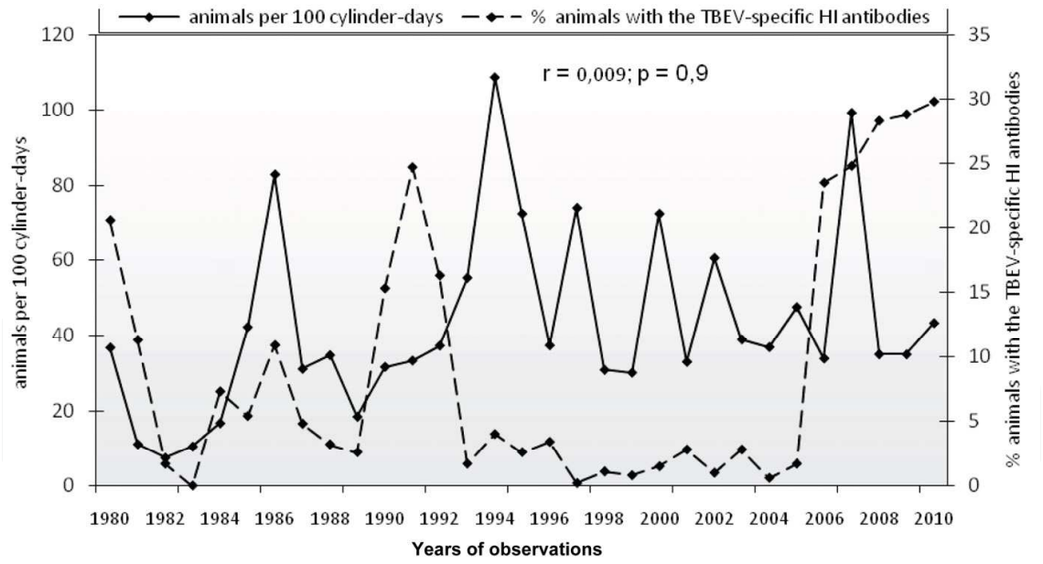


Fig. 7. Long-term dynamics of population density and HI antibody prevalence among small mammals in Novosibirsk endemic region, South-Western Siberia, Russia

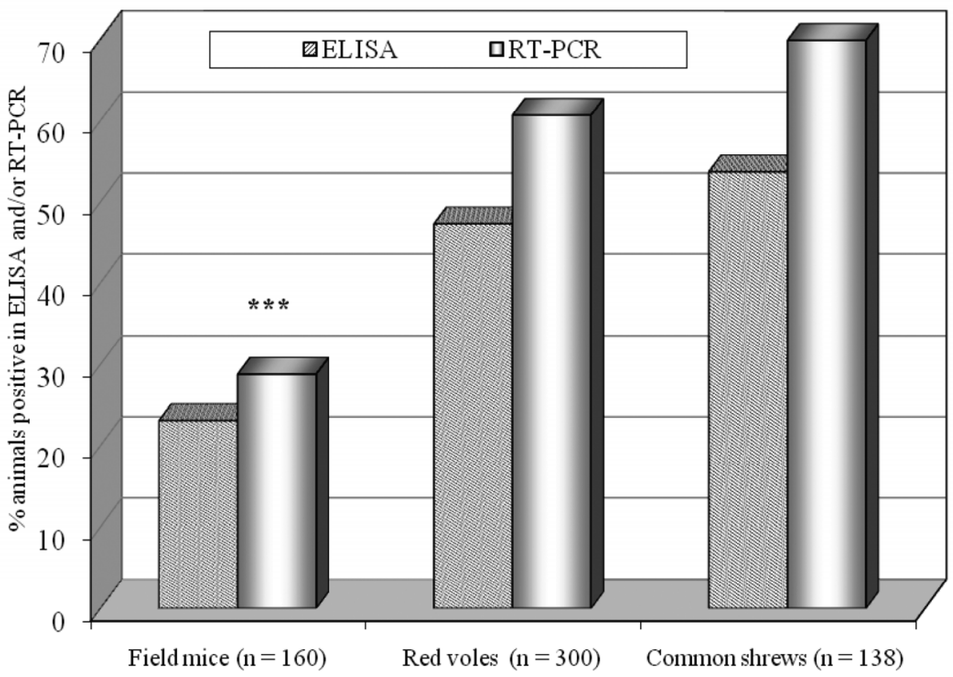
Analysis revealed cyclic variations of both small mammals abundance and HI antibodies prevalence (Fig.7). For TBE parasitic system both short (less than 7 years) and middle (more than 12 years) cycles were described (Naumov et al., 1984).

For proportions of immune hosts short cycles lasted during 3-7 years whereas middle – 12-19 years with HI antibodies prevalence less 4% for short cycles and from 0 to 28.8±4.5 % for

middle ones (Fig.7). For animal abundance middle cycles lasted for less than 14 years. One should note that proportion of immune hosts did not significantly correlate with the abundance of all small mammals (r=0,009; p=0,9), insectivorous (r= -0,6; p=0,7) or rodents (r= -0,8; p=0,67). However, the calculated correlation coefficients (r) allowed us to reveal rather slightly negative correlation. At population depression stages with decreasing amounts of small mammals the growth of proportion of immune animals might be caused by elevated tick infestation and/or activation of endogenous persisting TBEV.

Prevalence of HI antibodies coincided with ELISA-detected IgM antibodies typical for the virus reproduction periods. But IgG levels were essentially higher and corresponded to natural TBEV infection among wild small mammals (data not shown).

TBEV persistence among wild small mammals was studied using bioassay, HI test, ELISA and RT-real-time PCR with subtype-specific fluorescent hydrolysis probes or nested PCR with subsequent sequencing. Average TBEV prevalence for common shrews was 82.6±3.3%, for red voles – 71.7±2.6% and but for field mice – significantly lower 36.8±3.8% ($p<0.001$) in organs (Fig. 8) and in blood samples from red voles 71.2±5.9% and from field mice 41.3±7.3%.

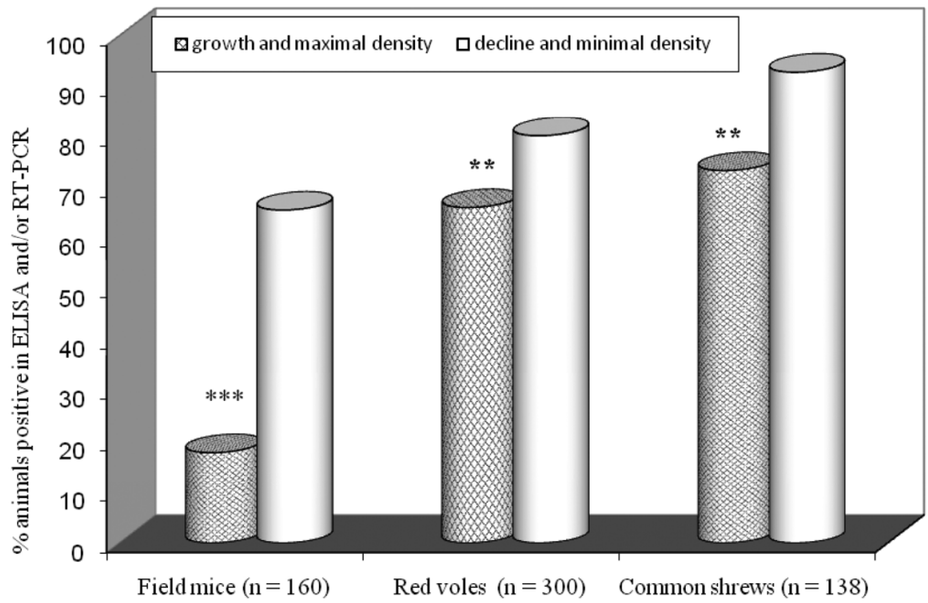


*** - p<0,001 – significance level for field mice compared to red voles and common shrews.

Fig. 8. Detection of TBEV RNA and glycoprotein E in organs of small mammals for 1998-2009

Despite high TBEV prevalence detected in ELISA and RT-PCR pathogenic in bioassay and hemagglutinating TBEV was found in a few samples of small mammal organs only. In 7-15 days postinfection of 1–2-day-old suckling mice with homogenates of organs from wild small mammals weak clinical manifestations of TBE such as languor, lack of appetite or refusal to eat, slight tremor of extremities were registered for mice of both first and second passages without subsequent death. Majority of TBEV strains isolated from wild small mammals were not stable in spite of detection of both TBEV RNA and protein E in organs of bioassay laboratory mice.

TBEV infection rate among wild animals were significantly lower ($p<0.001$ – $p<0.01$) at stages of their population growth and maximal density in comparison with periods of population decline and minimal density (Fig. 9).



*** - p<0,001; ** - p<0,01 – significance levels for each species.

Fig. 9. Detection of TBEV RNA and/or glycoprotein E in organs of wild small mammals (average values for 1998-2009) versus their population cycles

During cold seasons (October 16 – April 15) average TBEV infection levels were significantly higher ($p<0,001$) compared to warm periods (April 16 - October 15) for all 3 species of small mammals studied. Relative abundance of TBEV infected red voles remained high in any seasons of year whereas for field mice and common shrews more essential prevalence ($p< 0.01$; $p< 0.05$, respectively) were noticed in winter time (Table 1). Dependence of the TBEV infection rate on the mammal species, a season of an year or a population cycle phase could be caused by the different innate unspecific resistance of species of wild animals to infections, cold immunosupression and/or hormonal influence during reproductive cycles.

Species of animals	TBEV detection (%)		Student's criterion and significance levels
	Autumn-winter period	Spring-summer period	
Red voles *Myodes rutilus*	71,9±3,8 (100/139)	71,6± 3,9 (96/134)	-
Field mice *Apodemus agrarius*	61.1± 8.2 (22/36)	22.3± 4.3 (21/94)	t = 4.3; p< 0.001
Common shrews *Sorex araneus*	83.1±4.7 (54/65)	54.7± 7.8 (23/42)	t = 3.1; p<0.01

Table 1. Detection of TBEV RNA and glycoprotein E in organs of wild small mammals trapped in different seasons of year (data pooled 1998-2008)

Molecular typing of TBEV from organs and blood cells of wild small mammals was performed by RT-nested PCR with subsequent sequencing and phylogenetic analysis as previously described (Bakhvalova et al., 2006) and by RT-real-time PCR with TBEV subtype-specific fluorescent probes. Both Far Eastern, Siberian and European genetic subtypes with predominance of the first as well as mixed infection up to 20% were revealed in organs and blood cells of small mammals in Western Siberia, Russia. Siberian subtype prevalence and corresponding viral loads in blood of small rodents of 2 species were significantly ($p<0.01$ - $P< 0.001$) lower in comparison with Far Eastern subtype. Siberian subtype was detected mainly as mixed infection with Far Eastern subtype. Viral loads were similar for 3 species of small mammals studied. Threshold cycles (Ct) of the TBEV in organs of wild small mammals varied from 29 to 55 and allowed us to estimate the TBEV RNA quantities as 10^3-10^6 copies per brain or spleen (Fig. 5). In cells of 100 µl of blood the viral loads varied from a few copies to 10^2 for Siberian subtype and up to 10^7 - for Far Eastern subtype but in sera the TBEV RNA was not detected (data not shown). Thus, for small mammals high levels of life-long persistent infection with the TBEV of Far Eastern, Siberian and rarely European subtypes were observed in the presence of the virus-specific antibodies including HI- and virus-neutralizing antibodies (Okulova, 1986).

4. TBEV quasispecies genetic rearrangements in ticks and mammals

RNA viruses commonly exist as a swarm of viruses with varying genomes. The variations are predominantly the result of the lack of proof-reading capability in the virally-encoded RNA-dependent RNA polymerases, the production of large numbers of viral genomes and the absence of RNA repair systems in both host eukaryotic cells and in virus. Eigen and colleagues introduced the concept that this genetic heterogeneity could be described as a quasispecies, a "complex self-perpetuating population of diverse related entities that act as a whole" (For review see Biebricher and Eigen, 2006). Survival of the fittest is replaced by survival of the fittest quasispecies. In a quasispecies, the wild type is not a specific sequence, but consists of the weighted average of nucleotides at each position. However, the interpretation that genetic variability in RNA viruses should invariably be attributed to quasispecies has been questioned (Holmes and Moya, 2002). Most authors reporting sequence heterogeneity ignore these theoretical considerations and label any genetic variation in the same specimen as evidence for quasispecies. By repeated sequencing of the same specimen, quasispecies in flaviviruses have been well documented for dengue virus types 1 (DENV-1) (Aaskov et al., 2006) and 3 (DENV-3) (Chao et al., 2005) as well as for West Nile virus (WNV)(Jerzak et al., 2005) and TBEV (Romanova et al., 2007).

Phylogenetic analysis of E gene nucleotide sequences for TBEV RNA isolated from brain, liver, spleen and blood cells of small mammals using 4 alternative algorithms of Mega 4.1 software revealed Far Eastern and Siberian subtypes with predominance of the first (Fig. 10) Despite of the different genetic subtypes TBEV isolates from wild small mammals were notable for their low pathogenicity, immunogenicity and reproduction activity.

Analysis of the TBEV 3'UTR nucleotide sequences of chronological row of the TBEV strains isolated in Novosibirsk endemic region, South-Western Siberia, Russia during 1980-2010 revealed the presence of large, variable deletions located near oligo(A) fragments within hypervariable region located downstream from termination codon of a single open reading frame and numerous nucleotide substitutions (Morozova et al., 2007; GenBank accession numbers DQ473396-DQ473404). Lengths of the TBEV 3'UTR for isolates from ticks were

shorter than those from mouse brain suspensions after infection with corresponding tick homogenates. Large deletions did not arise only during serial passages with high multiplicity of infection as previously supposed but could be also found in primary isolates. The deletions were found in the 5′-terminal region of the 3′UTR, a region considered to be variable and, at least under laboratory conditions, not necessary for virus viability. Deletions may also occur in the 3′ end of the 3′UTR, a region that contains sequences and calculated structures essential for viral propagation. One of these is the cyclization sequence CGGUUCUUGUUCUCC. Another is a calculated long stable hairpin (3′LSH).

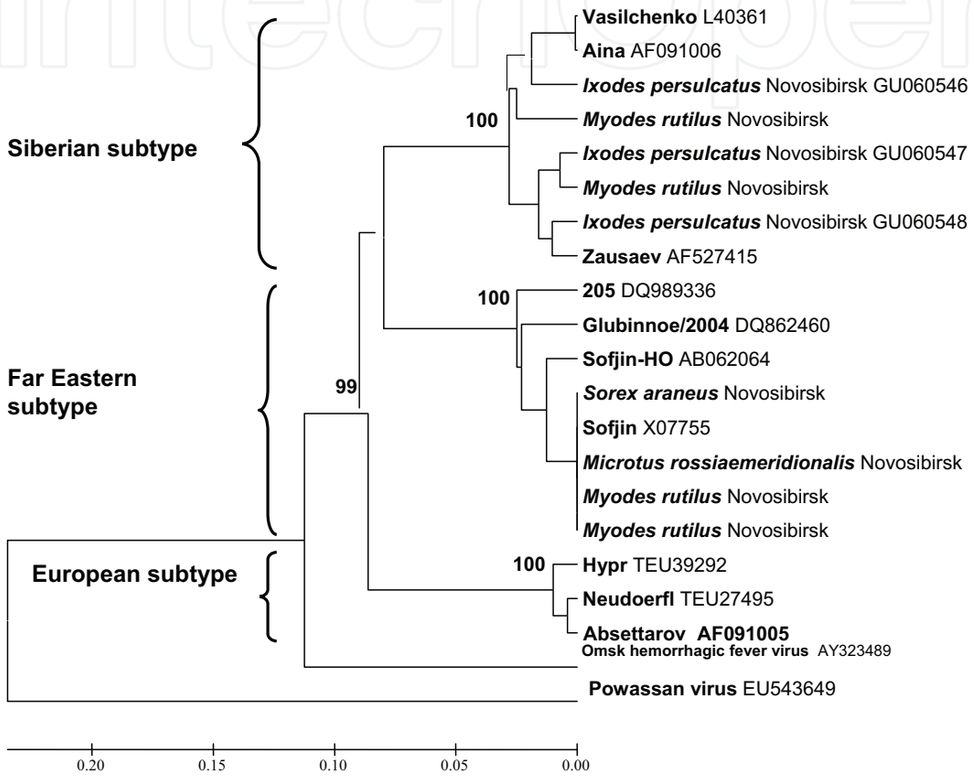


Fig. 10. Phylogenetic tree using UPGMA algorithm of Mega 4.1 software of nucleotide sequences of the TBEV E gene for the viral RNA isolated from ticks and wild small mammals from Novosibirsk endemic region, Western Siberia, Russia

Manual multiple alignment starting at the 3′ end of 3′UTR sequences of the TBEV strains from our collection revealed that the sequences could be divided into 3 groups. The first contained 712-730 nucleotides corresponding to full length 3′UTR. The second group contained deletions, principally in the 5′ end, but occasionally in the 3′ end that did not include deletions in the predicted cyclization sequence and usually not in the calculated 3′LSH. The third group contained deletions that included both the cyclization sequence and the entire 3′LSH. According to current concepts members of the second group might be viable, but the third group should not be. These findings suggest that viability in the third group that had characteristically been passaged multiple times was maintained by dual infection with fully viable virus. The results suggest the presence of sequence variability sometimes referred to as quasispecies. One should note that 3′UTR sequences obtained from small rodents and insectivorous naturally-infected with TBEV indicate a multiplicity of

electrophoretic bands, a finding that also supports the presence of quasispecies in TBEV. Perhaps, the variable fragment of 3'UTR might be responsible for binding with host cell replication factors thus permitting to select tick- or mammal-adapted variants of the virus quasispecies.

5. Periodic variations of TBE morbidity rate

In endemic regions TBEV transmission has been found to occur in one of about 200 tick bites. Moreover, tick bites are not often noticed and in the case histories references to tick bites are mentioned in 10-85% (Tick-Borne Encephalitis (TBE) and its Immunoprophylaxis, 1996). Maximal TBE morbidity rate had been registered in 1956 (5,163 cases) and 1964 (5,205 incidences). Then until 1974 TBE prevalence gradually declined to 1,119 cases. During 1976-1989 an average annual morbidity level in Europe and Russia was 2,755 and between 1990 and 2007 - an average of 8,755 reported cases of TBE per year (Mansfield et al., 2009). In 1999 11,356 cases of TBE in Eurasia (www.tbe-info.com) (and among them 9,955 – in Russia alone) render the highest sickness level in all endemic regions. However, these underestimations of TBE rate are based on confirmed hospitalized cases of severe encephalitis or meningoencephalitis and comprise nearly 20-30% of real TBEV infection prevalence among populations from endemic regions. The figures are second only to those for Japanese encephalitis (Mansfield et al., 2009 and references therein). An annual TBE vaccination campaign was introduced in Austria in 1981. Despite once having the highest incidence rate in Europe (up to 700 hospitalised cases annually), this campaign caused a steady decline in the number of cases of TBE in Austria alone. In surrounding European countries under similar climate conditions vaccination have had varying degrees of success, since the vaccines are expensive and must have repeated administrations in order to maintain protective immunity. For example, the Czech Republic still has one of the highest incidence rates in Europe, with 400–1000 clinical cases reported annually (Ruzek et al., 2008). In Russia TBE morbidity rate during last decade gradually decreased but it could not be attributed to vaccination with an average rate 5-7%.

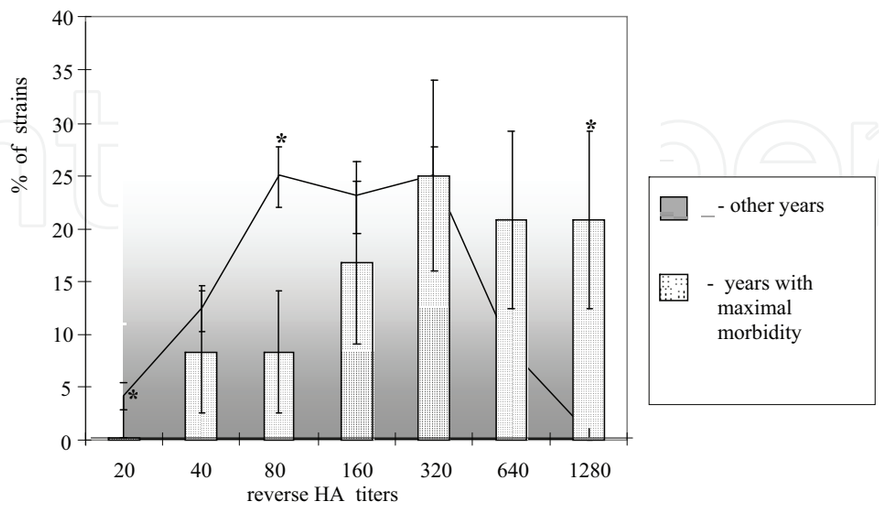


Fig. 11. HA titers of the TBEV strains during periods with different TBE rate in humans (* -P<0.05)

One of the striking epidemiological features of TBE is periodic variation in the occurrence and severity of TBEV infections in different endemic regions from Far Eastern Russia to Europe. Peak values last 1-2 years and trough values last for 6-7 years separated by intervals of gradual transition over 1-5 years. Three such cycles have been observed in the Novosibirsk region, South-Western Siberia, Russia spanning a period of 30 years of observations beginning from 1980. The TBE morbidity in Novosibirsk region periodically changed with maximal sickness rate in 1981 (47.7 incidences per 100,000 population), 1992 (60.7 incidences per 100,000) and 1999 (32.9 incidences per 100,000). The TBEV infection rate of the main arthropod vectors-ixodid ticks was previously shown to vary from 0.3 to 3.1 % according to bioassays (Fig. 1) and up to 46% based on RT-PCR. The TBEV strains isolated from ixodid ticks during period between 1980 and 2010 years in the same endemic region of Siberia, Russia were analyzed after 1-15 passages in susceptible laboratory suckling mice. Changes in TBE incidence in patients paralleled the viral hemagglutination titers and neuroinvasiveness indexes for the TBEV strains isolated from unfed ticks. The 3'UTR turned out to be one of the most variable parts of the genome. Sequencing revealed numerous 3'UTR rearrangements including microdeletions of 1-5 nucleotides located near oligo (A)-tracts 2-6 nucleotides long. The first group of the TBEV strains included the high-virulent strains isolated in 1982 and 1992 with high morbidity among people in Novosibirsk region whereas all the TBEV strains from the second group have been isolated during periods with relatively low sickness rate in the endemic region (1984, 1988 and 2006). Diversity observed for the 3'UTR sequences appeared to be not essential for the viability of the TBEV strains. Longer incubation period for more than 5 days in suckling mice observed for the TBEV strains with shorter 3'UTR fragment sequences (less than 200 nucleotides) in comparison with strains with longer (more than 300 bp) sequences (4 days incubation period) might be probably caused by slower rate of RNA replication. However, reduced neuroinvasiveness could not also be excluded as the reason for different incubation periods. Thus, structural rearrangements in the TBEV 3'UTR presumably did not significantly affect translation but probably could compromise RNA synthesis.

Hemagglutination titers varied from 1:20 to 1:1280 for the TBEV strains and did not correlate with length of 3'UTR fragment. However, cyclic variations of the TBEV HA titers coincided very well with periodic changes of the disease rate in humans (Fig. 11). Neuroinvasiveness indexes for 40 TBEV strains varied in a range 0.3 -4.3 (titers 5.8-8.5 lgLD50 after intracerebral infection and 2.7-7.0 lgLD50 after subcutaneous administration). It is interesting to note that in years with peak sickness rate in human's neuroinvasiveness indexes of the TBEV strains studied were higher than in other years.

Periodic variations in the incidence and severity of TBE have been shown to be inversely correlated with both adult tick abundance (A) and TBEV prevalence in ticks (B) (Fig. 12). At least three possibilities should be taken into consideration to explain the inverse association of these two parameters. One is that the changes are the result of differences in the proportion of infections caused by the three phylogenetically defined subtypes (Far Eastern, Siberian and European). A second possibility is that cyclic alterations in TBEV loads in arthropod vectors might be involved. Finally, TBEV quasispecies rearrangements including variable ratio of both tick-adapted and mammal-adapted variants cannot be exluded . The 3 possibilities could be interrelated.

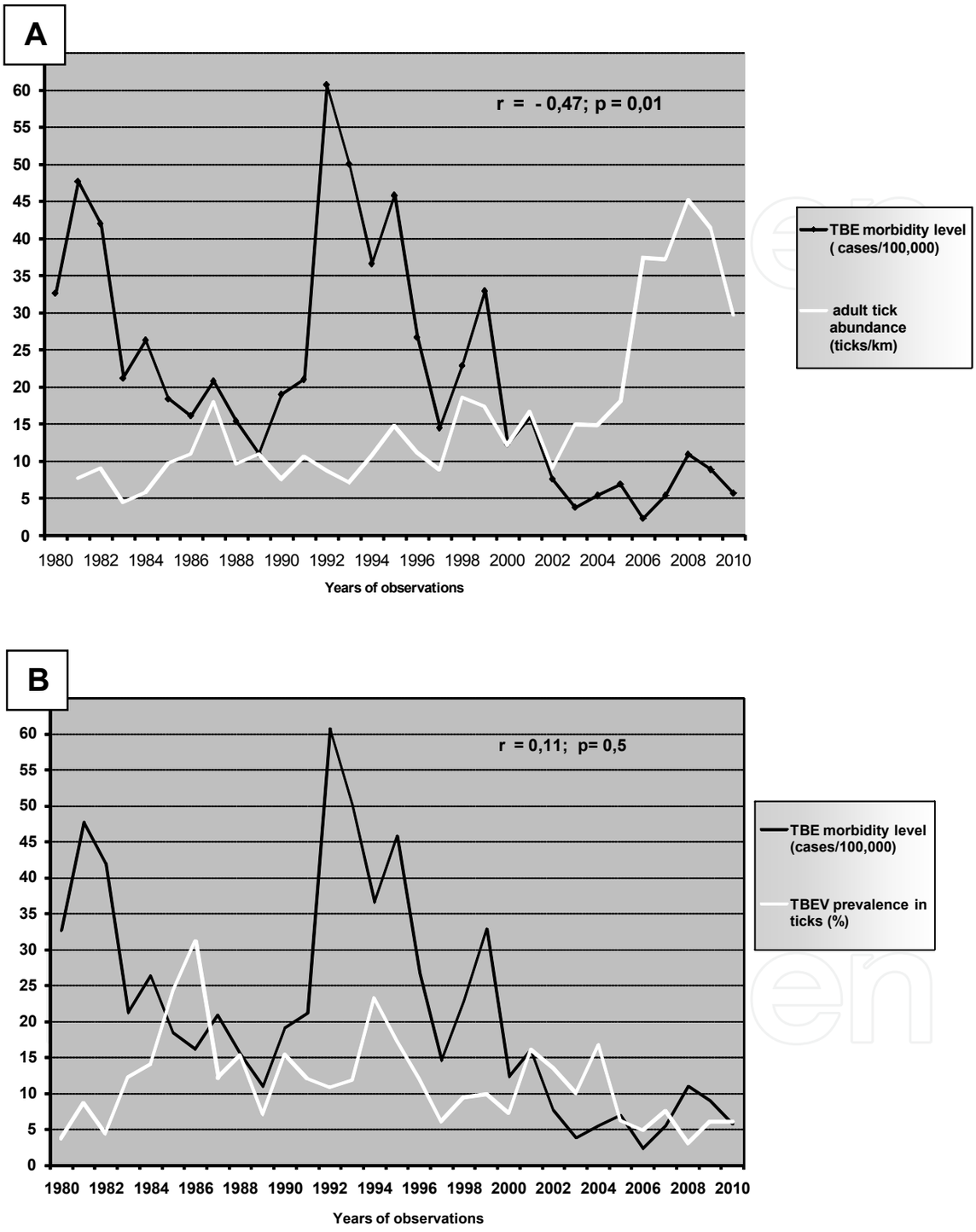


Fig. 12. Reverse correlation between TBE morbidity rate, adult tick abundance (part A) and TBEV prevalence in ticks (part B) in Novosibirsk endemic region, South-Western Siberia, Russia

6. Conclusion

Taken together, our long-term observations of the TBEV in its natural hosts in the endemic region of Western Siberia, Russia revealed the followings.

1. Essential differences of the TBEV infection rate and the viral loads were observed between different reservoir hosts (Fig. 2, 5, 7-9, table 1). The TBEV prevalence among ixodid ticks was significantly lower compared to the corresponding infection rate among vertebrate hosts, whereas the viral loads in a tick was in 10-100 times higher than in organs and blood cells of mammals. However, pathogenic TBEV could be easily isolated from ticks but hardly only in a few cases from mammals. Both Far Eastern, Siberian and European genetic subtypes were identified in the virus isolates from reservoir hosts but in different proportions. One should note the selection of Siberian genetic subtype in brains of laboratory suckling mice used for bioassay that might distort a real structure of the TBEV quasispecies in wild hosts. Specific features of the TBEV in different reservoir hosts might be the consequences of a long adaptation resulting in the resistance of the whole parasitary system.
2. The TBEV natural population (Fig. 2, 11) together with the reservoir hosts of the virus (Fig. 1 and 6) periodically changed as the whole system. For periods of isolation of the TBEV strains with high hemagglutination (HA) titers (≥1:320) (Fig. 11) and elevated geometric middling (Fig. 2) the TBE morbidity rate in the endemic region was also maximal (Fig. 11, $r=0,63$, $p\leq0,05$). The changes of the TBEV HA activity in ticks correlated with dynamics of small mammals with HI antibodies ($r=0,69$; $p\leq0,01$) with interval of 1 year required for transstadial transmission of the TBEV from immature ticks to adults and, consequently, with the viral encephalitis rate ($r=0,49$; $p\leq0,001$).
3. Cyclic changes of the TBEV infection rate in ticks as well as tick abundance (Fig. 12) did not correlate with TBE morbidity rate in the same endemic region.

7. Acknowledgments

Current work was supported in part by integration projects №19, №83 of Siberian Branch of the Russian Academy of Sciences.

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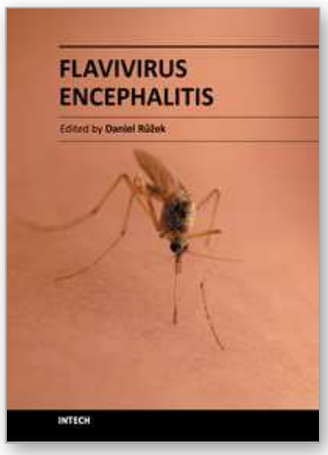

Flavivirus Encephalitis

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.

How to reference

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Bakhvalova V.N., Panov V.V. and Morozova O.V. (2011). Tick-Borne Encephalitis Virus Quasispecies Rearrangements in Ticks and Mammals, Flavivirus Encephalitis, Dr. Daniel Ruzek (Ed.), ISBN: 978-953-307-669-0, InTech, Available from: http://www.intechopen.com/books/flavivirus-encephalitis/tick-borne-encephalitis-virus-quasispecies-rearrangements-in-ticks-and-mammals

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