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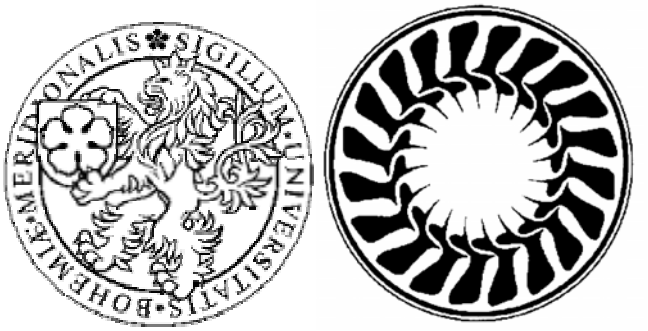

MOLECULAR ASPECTS OF EPIDEMIOLOGY AND PATHOGENESIS OF TICK-BORNE ENCEPHALITIS

Dissertation Thesis
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Philosophiae Doctor (PhD.)

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presented by

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Annotation:
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Abstract:
The dissertation thesis is focused on the investigation of molecular epidemiology and pathogenesis of tick-borne encephalitis. The thesis includes studies on molecular evolution of tick-borne encephalitis virus in Central Europe since the discovery of the virus up to the present (1948-2002), investigation of the microevolution of the virus in laboratory animals and cell cultures, interaction of the virus with cell lines derived from various vector and non-vector ticks, and studies on the pathogenesis of the disease with special regard to the role of host immune system in the development of encephalitis and to the interaction of the virus with neural cells.

Abstrakt:
Předkládaná disertační práce je zaměřena na studium molekulární epidemiologie a patogeneze klíšťové encefalitidy. Práce zahrnuje studie týkající se molekulární evoluce viru klíšťové encefalitidy (KE) ve střední Evropě od prvního objevení viru v roce 1948 až po současnost. Dále se zabývá mikroevolucí viru v buněčné kultuře a v laboratorních zvířatech, studiem interakce viru KE s buňkami odvozenými od různých vektorově kompetentních a nekompetentních klíšťat a v neposlední řadě studiemi zaměřenými na patogenezi klíšťové encefalitidy se zvláštním zřetelem na úlohu hostitelského imunitního systému při rozvoji encefalitidy a na interakci viru s lidskými neurálními buňkami.

Prohlašuji, že jsem svou disertační práci vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění, souhlasím se zveřejněním své disertační práce, a to v nezkrácené podobě – v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou - elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stánkách.

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"Attitudes are more important than abilities.
Motives are more important than methods.
Character is more important than cleverness.
Perseverance is more important than power.
And the heart takes precedence over the head."

Dr. Denis Burkitt

To my family and my teachers.

Content

Chapter I.
Introduction and literature review

I.1 Introduction

Research during the past few decades has yielded considerable progress in many areas of the flavivirus problematic, but there remain a number of enigmatic challenges surrounding our understanding of the behavior of flaviviruses to their invertebrate as well as vertebrate hosts. In conjunction with advances in flavivirus molecular virology, more and more attention is being directed to the investigation of the epidemiology and pathogenesis of flavivirus encephalitis. The pathogenesis of the diseases caused by flaviviruses, including tick-borne encephalitis (TBE) as the most important tick-borne viral disease in Europe and Central and Northern Asia, has been the subject of many classical studies, mostly in the 60s and 70s of the last century. However, the research possibilities have now progressed to the molecular level as well, thanks to the current availability of the broad spectrum of modern molecular technologies, allowing facing to the major remaining problems in the understanding of flavivirus encephalitis.

I.2 Goals of this project

The aim of this project was to investigate the epidemiology and pathogenesis of TBE with the application of modern molecular techniques.

(i) The first part of the project is focused on the molecular epidemiology of TBE in the Czech Republic, since the time of the discovery of the virus in Europe up to the present with the purpose to determine the structure of viral genetic diversity in the region of Central Europe and the evolutionary processes occurring within the virus. We analyzed molecular characteristics of strains from different time periods and different sources that included human samples, ticks and natural hosts. The molecular analysis was based on the complete sequence analysis of envelope (E) gene and 3' non-coding region. Determined amino acid substitutions in the E protein of the analyzed strains was superimposed on the 3D structural model of the protein and discussed with the respect to the antigenic and virulence variations during the last 50 years.

(ii) Molecular-epidemiological studies of circulation of the TBEV subtypes in different areas of Eurasia are unconceivable without a simple and reliable method of subtyping of tick-borne encephalitis virus (TBEV) isolates. For that reason, we developed a rapid and simple method based on multiplex RT-PCR for subtyping of TBEV isolates.

(iii) Next to this, we analyzed interaction of the virus with tick cells (comparison of the growth of the virus in tick cell lines derived from different vector and non-vector ticks). We examined the susceptibility of tick cell lines derived from five ixodid and one argasid tick species to infection with a representative strain of TBEV, with special regard to tick species living outside the areas of occurrence of TBE, which have not been reported to transmit the virus. The aim was to evaluate the potential vectorial capacity of such ticks for TBEV in comparison to the principal European vector *I. ricinus* at the cellular level. With regard to global climatic changes, changes in agricultural production and leisure time activities and increased travel, we can expect a future shift in the abundance, dynamics, and geographical distribution of ticks and tick-borne viruses, allowing previously unexposed tick species to come into contact with TBEV.

(iv) In the next part of the project, we studied the microevolution of an attenuated temperature-sensitive strain of tick-borne encephalitis virus, during serial subcultures, either 5 times in mice, or at 40ºC in PS cells. Developed variants were analyzed with respect to their biological properties and full-length genome analyses were performed. This study led to the discovery of molecular determinants of virulence, specific mutations that affect mouse neuroinvasiveness and determine temperature-sensitive phenotype of the virus.

(v) Pathogenesis of TBE was investigated in two separate studies. Firstly, we analyzed the role of host immune system in TBE. We addressed this issue by analysing the role of specific T-cell subpopulations, i.e. $CD4^+$ and $CD8^+$ T-cells, in the recovery and/or immunopathology of TBE in mice. Secondly, we studied the interaction of TBEV with human neural cells. Despite the fact that TBEV is highly neurotropic, little work has been done with human cells of the neural origin. In this study, TBEV was used to infect three human neural cell lines: neuronal, medulloblastoma and glial cells. The susceptibility and virus-mediated cytopathic effect including ultrastructural and apoptotic changes of the cells were deeply examined.

I.3 Tick-borne encephalitis

I.3.1 Historical consequences

Although the first hints of the existence of tick-borne encephalitis date back to the 18th century (Aland islands, Finland), the first medical description of the disease was given by Austrian physician H. Schneider in 1931 (Schneider, 1931). In 1937-39, Russian Ministry of Health organized three successive expeditions to the Far East with the purpose to elucidate the origin of severe infections of the central nervous system, called "Taiga encephalitis", disease that had been observed there since 1914, but more frequently occurred since 1933. The expeditions revealed viral origin of the disease and the tick *Ixodes persulcatus* as the main vector of the virus (Zilber, 1939). The disease was called 'Russian spring-summer encephalitis' (or Far East or taiga encephalitis); the virus become known as Russian spring-summer encephalitis virus and lately tick-borne encephalitis virus.

In Europe, tick-borne encephalitis virus was first isolated from human patients in Czechoslovakia after the Second World War in 1948 (Gallia et al., 1949; Krejčí, 1949b) when the incidence of clinical manifestations caused by the virus was so high, that it was noticed by infectologists in affected regions (Krejčí, 1949a). Simultaneously, the virus was isolated also from ticks *Ixodes ricinus* suggesting the role of the tick as a vector of the disease (Rampas and Gallia, 1949).

Retrospective analysis, however, revealed the presence of clinically similar disease not only in Czechoslovakia, but also in a number of other European countries for several decades before the first isolation (reviewed in Izbický, 1954). In Czechoslovakia, this disease was previously known as '*Encephalitis epidemica'*. Since 1945, there was nearly tenfold increase in the incidence of this disease associated with decrease in mortality (mortality of 50.8% in 1936-1944, 17.8% in 1946; Izbický, 1954).

Shortly after the description of TBE in Czechoslovakia, the virus was isolated in Hungary (Fornosi and Molnár, 1952), Poland (Przesmycki et al., 1954; Szajna, 1954), Bulgaria (Vaptsarov and Tarpomanov, 1954), Yugoslavia (Bedjanič et al., 1955), Austria (Pattyn and Wyler, 1955), Romania (Draganescu, 1959), eastern Germany (Sinnecker, 1960), but also in Finland (Oker-Blom, 1956) and Sweden (Kaäriainen et al., 1961). Simultaneously, the virus was also revealed in China and Japan (Ando et al., 1952).

The clinical course of the disease, its pathology and epidemiology, as well as the properties of the virus, its ecology and ecology of the vectors have been studied in detail. Most of the studies were carried out in Russia, Czechoslovakia and Austria (reviewed in Kunz and Heinz, 2003).

I.3.2 Virus

The virions are spherical particles, approximately 50-60 nm in diameter (Slávik et al., 1967) with a nucleocapsid composed of a (+)ssRNA genome enclosed in a capsid (C) protein and surrounded by a host cell-derived lipid bilayer. Two virus proteins are integrated in the lipid membrane, the envelope (E) protein and the membrane (M) protein (**Fig. 1A, B**).

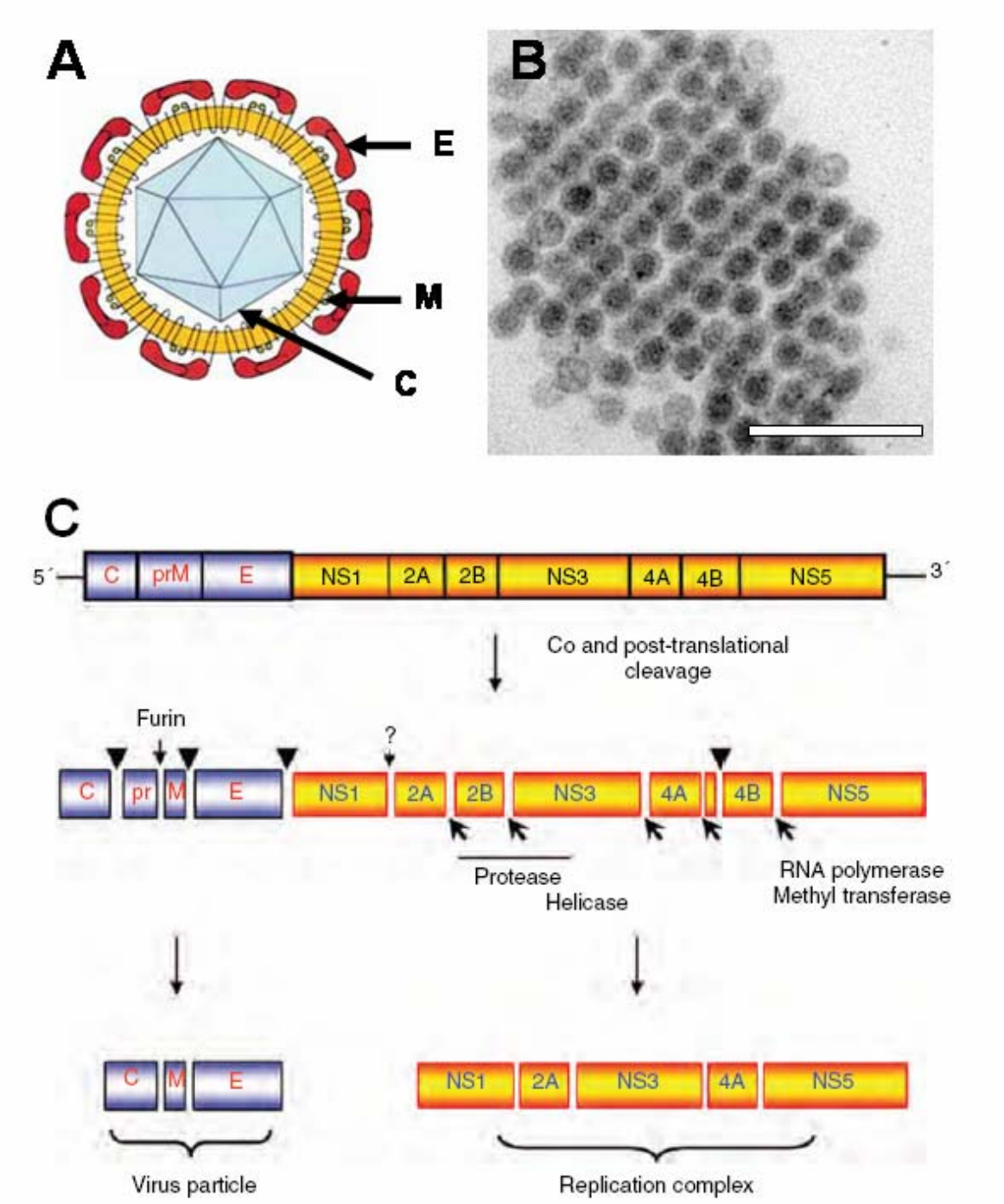

Fig. 1. (A) Structure of mature TBEV virions. Mature virions contain E and M proteins on their surface. E is present as homodimeric complex (from www.tbe-info.com). (B) Electron micrograph of TBE virus particles. Bar represents 200 nm (orig.). (C) Schematic representation of the flavivirus genome organisation and polyprotein-processing events associated with replication. Cleavage sites depicted with ▼ represent cleavage by host signal peptidase; sites depicted with ↖ represent cleavage by viral NS2B-NS3 protease (adapted from Mackenzie, 2005).

The (+)ssRNA genome is approximately 11 kb in length and contains one large open reading frame (ORF) which is flanked by 5' and 3' untranslated regions (UTR). The genome carries a 5'-cap and no 3'-poly(A) tail (**Fig. 1C**). The untranslated regions form secondary stem-loop

structures that probably serve as *cis*-acting elements for genome replication, translation and/or packaging (Gritsun et al., 1997, Proutski et al., 1997a,b). The ORF encodes a single polyprotein that is cleaved by viral and cellular proteases into three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (reviewed in Lindenbach and Rice, 2003).

C (capsid) protein is a relatively small basic protein with lower sequence homology between different flaviviruses (Lindenbach and Rice, 2003). The carboxyl terminus of C protein serves as an internal signal sequence leading the structural protein prM into the membrane of endoplasmic reticulum (ER). The viral protease NS2B-NS3 cleaves this signal sequence, releasing the N-terminus of prM protein (Kofler et al., 2002). prM protein is a glycosylated precursor of the membrane protein M. The prM protein shows a chaperon-like activity during the envelope protein E folding (Lorenz, 2002; Lorenz et al., 2002).
Viral envelope (E) protein is the major antigenic determinant of the virus and is responsible for specific binding to a cellular receptor and penetration of the virus into the host cell. It is supposed to be a main determinant of the virulence (Gritsun et al., 1995). Three-dimensional structure (**Fig. 2**) of the protein E was studied at the resolution of 2.0 Å by X-ray crystallography (Rey et al., 1995).

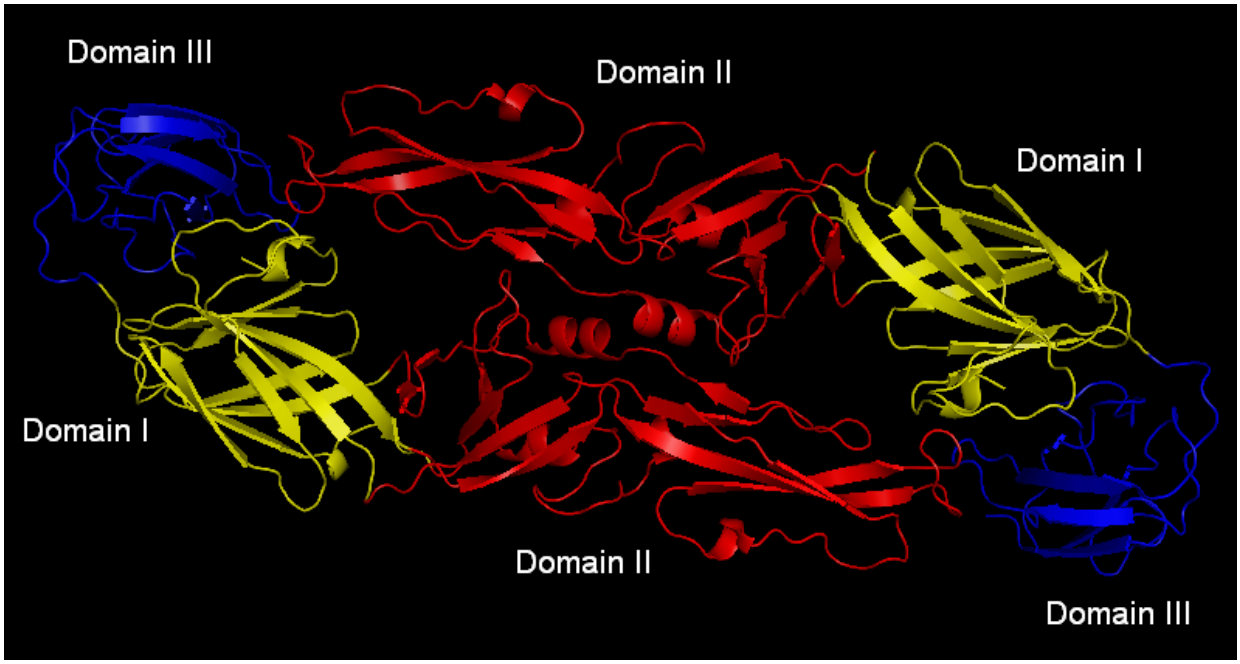

Fig. 2. Diagram showing the folded structure of TBEV E polypeptide chain in the dimer. The predicted three-dimensional structure (1svb.pdb) was produced using PYMOL facilities available at http://pymol.sourceforge.net/ (orig.).

The protein is formed by two monomeric subunits anchored in the membrane by their distal parts at physiological pH. In the environment with acidic pH (e.g. in endocytic vesicles), irreversible changes in its structure take place including its re-arrangement to trimeric forms. This conformational change plays a significant role during the fusion of viral envelope with the membrane of endosome formed at the entry of virus into the cell (Holzmann et al., 1995).
Each monomeric subunit is composed of three domains (I-III). Domain I is located in the central part of the protein. It is formed by eight antiparallel beta sheets, contains the N-terminus of the protein, two disulfide bridges and an N-glycosylation site. The E protein is glycosylated in the vast majority of flaviviruses. The protein is N-glycosylated in a single

position (Winkler et al., 1987) even if its amino-acid sequence contains also another glycosylation site. A biantennal glycan of complex type with terminal D-galactose and with L-fucose bound to the first N-acetyl-D-glucosamin was identified in virions replicated in chicken embryos (Grubhoffer et al., 1990). However, many experimental data indicate that the glycosylation is not essential for the function of the protein (reviewed in Chambers et al., 1990a; Hurrelbrink and McMinn, 2003). On the other hand, some studies on dengue (Mondotte et al., 2007) or West Nile virus (Hanna et al., 2005) indicate the necessity of the glycosylation for virulent properties of the virus. Domain II is considered to be a subdomain of the domain I. It is formed by two long loops that extend out of domain I and form a finger-like structure. The domain contains a number of beta sheets and three disulfide bridges (Rey et al., 1995; Heinz, 2003). Part of the protein responsible for the fusion of viral envelope with the membrane of the endosome – a so called fusion peptide – can be found within this domain (Heinz and Allison, 2003).
The domain III has the typical fold of an IgC molecule (Heinz, 2003). It contains a beta barrel composed of seven antiparallel beta sheets. It is supposed that the lateral part of the domain III is responsible for binding to a specific cellular receptor (Rey et al., 1995).
Amongst the most conserved parts of the E protein, there are 12 cysteine residues forming six disulfide bridges with conserved localization in comparison with all known flaviviruses (Nowak and Wengler, 1987).
There are three main antigenic domains, named A, B, and C. The A domain is localized in structural domain II, B in domain III, and C is situated in structural domain III. The A domain bears the epitopes common for different flaviviruses, while epitopes on the other two domains account for type and subtype specific reactivity (Rey et al., 1995).

Non-structural protein 1 (NS1) is cleaved from the polyprotein by a signal peptidase (Ryan et al., 1998). NS1 is a glycoprotein containing two or three potential glycosylation sites (Lee et al. 1989) with not exactly defined function. It exists in dimeric forms localized freely in the cytoplasm or associated with membranes. Besides, this protein is also secreted into the extracellular space particularly as a pentamer or hexamer and occasionally as a dekamer or dodekamer (Crooks et al., 1994). Therefore, this protein was previously called “soluble antigen”, and induces protective immune responses against flaviviruses (Gould et al., 1986).
NS2A is a small, hydrophobic protein with not defined function. It is supposed, that it plays a role in the forming of replication complex (Lindenbach and Rice, 2003). A small membrane-associated protein NS2B serves as a crucial cofactor for protease activity of the NS3 protein (Yamschikov and Compans, 1995). The central hydrophilic domain of the NS2B protein possibly interacts with the NS3 protein and it is flanked by hydrophobic regions probably anchored in the membrane (Chambers et al., 1993).
NS3 is the second largest viral protein. It contains conserved regions important for the function of the NS3 as a serine protease, helicase and RNA triphosphatase (reviewed in Lindenbach and Rice, 2003).
NS4A and NS4B are small, hydrophobic proteins. NS4A is probably part of the replication complex (Uchil and Satchidanandam, 2003), while the function of NS4B is not known.
NS5 is the largest and highly conserved viral protein serving as a viral RNA-dependent RNA polymerase (Steffens et al., 1999). Its C-terminus shares a sequence homology with RNA-dependent RNA polymerases of other (+)RNA viruses (Lindenbach and Rice, 2003).

I.3.3 Replication cycle

Many processes within TBEV replication cycle are poorly understood. A basic schema of TBEV replication within mammalian cells is depicted in **Fig. 3.**

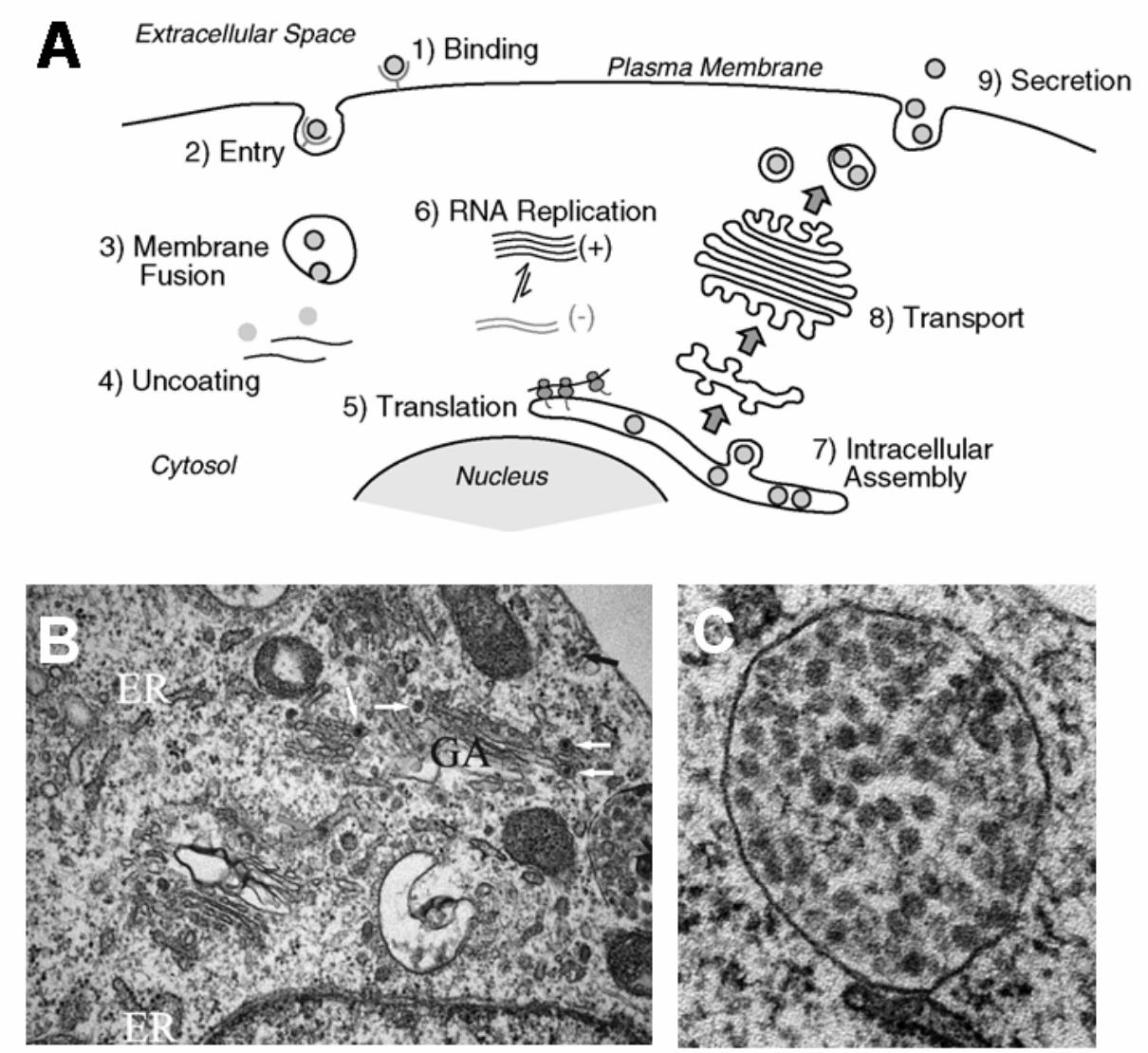

Fig. 3. TBEV life cycle in mammalian cell. (A) Schematic representation of steps in flavivirus lyfe cycle (adapted from Lorenz, 2002). (B) Electron micrograph of the development of TBEV in glioblast. Virus is present in the lumen of Golgi network (white arrows). (C) Vesicular transport of new virions in glioblast cell (orig.).

The infection of the host cell begins with the binding of the virus to a cell receptor, which has not been sufficiently identified till now. Kopecký et al. (1999) identified two polypeptides of 35 and 18 kD as putative vertebrate receptors for TBEV using viroblot technique with anti-idiotypic monoclonal antibodies directed against antibodies that neutralise the infectivity of TBEV. However, only one of the anti-idiotypic monoclonal antibodies bound weakly to the tick cells (line RA-257) (Kopecký et al., 1999). It is not clear whether TBEV use single or multiple receptors on susceptible cells. The results by Kopecký et al. (1999) indicate that different receptors are used by vertebrate and invertebrate cells for the binding of TBE virus. Apparently, just the ability to use multiple receptors can be responsible for the very wide host range of flaviviruses, which replicate in arthropods and in a broad range of vertebrates.
After binding to the receptor, the virus is internalized by endocytosis. Acidification of the interior of the endosomal vesicle changes the conformation of the E protein and rearranges its dimers to trimeric forms. These changes result in fusion of the viral envelope and the membrane of the endosomal vesicle (Holzmann et al., 1995) and the release of the viral

nucleocapsid into the cytoplasm. After uncoating, translation of the positive-stranded genome occurs, in parallel with synthesis of minus-strand RNA that serves as template for RNA replication. Processing of the polyprotein yields the individual viral proteins. The surface structural proteins prM and E are translocated into lumen of the ER and their amino termini are liberated by though proteolytic cleavage by host signalase. The newly synthesized RNA is packaged by protein C into nucleocapsids on the cytoplasmic site of ER. Viral envelope is acquired by budding of the nucleocapsid into ER. The immature non-infectious virions containing proteins prM and E in heterodimeric association are transported into Golgi network, where celavage of prM and reorganization of E-protein to the form of fusion-competent homodimers takes place. These mature virions are finally released from the host cell by fusion of the transport vesicle membrane with cytoplasmic membrane (reviewed in Mandl, 2005).
However, the TBEV maturation process in tick cells is completely different from the cells from vertebrate hosts. In cell line derived from the tick *Rhipicephalus appendiculatus* infected with TBEV, nucleocapsids occur in cytoplasm and the envelope is acquired by budding on cytoplasmic membrane or into cell vacuoles (Šenigl et al., 2006).

I.3.4 Classification

According to the recent taxonomy approved by the International Committee for Taxonomy of Viruses (Thiel et al., 2005), TBE virus is a member of the family *Flaviviridae*, genus *Flavivirus*, and is subdivided into 3 subtypes: European, Far Eastern and Siberian (Ecker et al., 1999). Encephalitis caused by members of the European subtype is usually mild with case fatality rate reaching 1-5 % (Grešíková and Kaluzová, 1997; Herzig et al., 2002; Zoehrer et al., 2003). On the other hand, Far Eastern strains produce severe encephalitis often with fatal outcome (Dumpis et al., 1999). TBEV strains from Siberian region cause a less severe disease, but with a tendency to develop chronic infections (Gritsun et al., 2003a). Generally, it is not very well known that Russian spring–summer encephalitis, Far Eastern encephalitis, Taiga encephalitis, Biphasic milk fever, Central European encephalitis, Kumlinge disease, and Frühsommer-Meningoenzephalitis are only regional names related to the same disease, caused by TBEV (Kunz and Heinz, 2003).
Recently, taxonomic revision of TBEV was proposed (Grard et al., 2007). Tick-borne encephalitis and Louping ill viruses are, despite their different biological properties, assigned to one species 'Tick-borne encephalitis virus', including four types: Western TBEV, Eastern TBEV (including Far Eastern and Siberian subtypes), Turkish sheep TBEV (including Greek goat encephalitis virus subtype) and Louping ill TBEV (including Spanish, British and Irish subtypes) (Grard et al., 2007). The relation of Louping ill virus to TBEV was subject of discussion since the first isolation of TBEV in Russia (Shubladze, 1958). A number of authors including Hubálek et al. (1995) demonstrated that TBEV and Louping ill are related on the basis of antigenic similarity and that the European TBEV subtype is closely related to Louping ill virus than to the Far Easten and Siberian subtypes (Hubálek et al., 1995).
Louping ill is transmitted to vertebrate hosts by *Ixodes ricinus* and causes a fatal encephalomyelitis in sheep and red grouse (*Lagopus lagopus scoticus*). Human infections caused by this virus are rare and benign.

I.3.5 Ecological and epidemiological characteristics

TBE occurs in many parts of Western and Central Europe, Scandinavia and Northern Asia, particularly, in Austria, Croatia, Czech Republic, Estonia, Finland, Germany, Hungary, Japan, Latvia, Lithuania, Poland, Russia, Slovak Republic, Slovenia, Sweden and Switzerland and also new TBE foci are emerging in a number of other European countries (Bröker and Gniel, 2003).

According to the concept of Pavlovskij, TBEV is maintained in a cycle involving ticks and wild vertebrate animals in forested natural foci under certain botanical, zoological, climatical and geo-ecological conditions (Pavlovskij, 1939). Ticks live preferentially in the dense undergrowth of the forests where the relative humidity is high.
The epidemiology of TBE is closely associated with the ecology and biology of ticks, the areas of their dissemination and periods of feeding activity. The maximum of human infections coincides with seasonal peaks of feeding activity of ticks. Peak feeding times for *I. ricinus* occur between May and June and also between September and October. On the other hand, *I. persulcatus* has only one peak of feeding activity between May and June (reviewed in Süss, 2003).
The incidence of TBE varies from year to year in different geographic regions. Across Eurasia, more than 10,000 human cases are reported annually. Over the last two decades, the most dramatic changes of all were the sudden increases (2- to 30-fold) in 1992-3 in Latvia, Lithuania, Poland and Belarus, with marked but lesser increases in Estonia, Germany, Slovakia, and the Czech Republic. TBE cases have increased steadily since the mid-1970s in Russia, and since the mid-1980s in Switzerland, Sweden, and Finland. In Austria, the only country with extensive systematic vaccination coverage, TBE incidence has decreased progressively since the early 1980s (Randolph, 2002).
In the Czech Republic, the incidence of the illness noticeably varied during the monitored period, i.e. since 1950s. The high occurrence of the infection in 1960s gradually decreases and in 1970s and 1980s reached value of 139-400 cases annually. Sporadically, there were more than 400 cases per annum in 1970, 1973, and 1979. A steep increase took place in 1990s (according to data from EPIDAT, National Institute of Public Health – www.szu.cz), when the annual incidence was more than twofold higher in comparison with preceding period, 400-600 cases per annum with maximum of 745 cases in 1995 and 706 in 2000 (Daniel et al., 2004). In the year 2006, the incidence (1,026 cases) of TBE in the Czech Republic was almost twice as high, than in the proceeding years, the highest ever recorded, indicating significantly increased epidemic activity of this important human pathogen (Daniel et al., 2008). A similar increase in number of cases was observed also in other regions of Europe (www.tbe-info.com/epidemiology/). This phenomenon is not definitely explained. One of hypotheses dealing with the increased incidence of the last years is based on impact of climatic changes on the biology of the vector *Ixodes ricinus*. Gradual raise of the temperature in last decades caused prolongation of the period of the tick development within a year and subsequently accelerating of its development and increase of the density of its population (Daniel et al., 2004). This allowed the intensification of the circulation of TBE virus, more frequent contact of man with infected tick, and caused dissemination of the tick and TBE to regions with no or rare previous records of their incidence (Daniel et al., 2003, 2006).
The temperature can affect also the circulation of the virus – the rate of virus propagation in the tick, the infection and transmission rates, as it is known for mosquito-borne viruses and as it is suggested from laboratory experiments with ticks (Danielová et al., 1983; Danielová, 1990). Moreover the transmitted pathogen can be affected by the temperature through the tick. Further on, the virus can be affected by a variety of selection pressures such as the rate and the mode of circulation (horizontal, vertical, non-viremic transmission).

I.3.6 Microevolution of TBEV

It has been recognized since the 1960s, that TBEV isolated from field-caught ticks contains a heterogeneous population of variants that produce a range of plaque sizes, express temperature-sensitivity, and neuroinvasiveness (Mayer and Kožuch, 1969).
The relatively rapid phenotype change of TBEV was first mentioned, when the TBE virus passaged in ticks *Hyalomma plumbeum* noticeably decreased the virulence for laboratory

mouse and subsequent passaging through mouse brains led to restoring of the former phenotype (Dzhivanyan et al., 1988). Similarly, passaging of TBE virus through salivary glands of ticks *Ixodes ricinus* led to reduction of neuroinvasiveness (Labuda et al., 1994). Analogously, the attenuated phenotype of the passaged virus was not stable; when the attenuated virus was passaged through mouse brains, the virulent phenotype was restored and after additional passages, the virus was more virulent than the parental strain (Kaluzová et al., 1994).

A Siberian strain isolated from *I. persulcatus* and passaged in mouse brain was subsequently passaged in *H. marginatum* by artificial inoculation and than again through mice. The tick-adapted virus exhibited small-plaque phenotype and slower replication in pig embryo kidney cells, higher yield in ticks, and decreased neuroinvasiveness in mice. A total of six amino acid substitutions distinguishing genomes of the variants were identified and two of them located in the E protein are supposed to be responsible for the phenotypic differences (Romanova et al., 2007).

In another study, an attenuated temperarute-sensitive TBEV strain (263), isolated from field ticks *I. ricinus*, was either serially subcultured, 5 times in mice, or at 40ºC in PS cells, producing 2 independent strains, 263-m5 and 263-TR with identical genomes; both strains exhibited increased plaque size, neuroinvasiveness and temperature-resistance. Sequencing revealed two unique amino acid substitutions, one mapping close to the catalytic site of the viral protease NS2B-NS3 (Růžek et al., 2008b).

The selection, during serial passage, of pre-existing quasispecies was postulated to be the explanation for the rapid shift of virus phenotypic characteristics. All the results lead to the suggestion that TBE virus exists as a heterogeneous population that contains virus variants most adapted to reproduction in either ticks or mammals. Host switch results in a change in the ratio of these variants in the population (Romanova et al, 2007). In other words, virulent and attenuated viruses may co-exist as quasispecies in the same TBEV population and rapid conversion of neurovirulence during virus tick/mammal adaptation is mediated by selection from the quasispecies population rather than random mutagenesis during virus passage in the laboratory (Růžek et al., 2008b; Chapter II.4).

I.3.7 TBEV in ticks

The principal vector of the European TBEV subtype is the tick *Ixodes ricinus* (Rampas and Gallia, 1949), a dominant hard tick across Europe. The virus infects ticks chronically for the duration of their life and circulates between ticks and their vertebrate hosts. However, the virus can be also transmitted transovarially (Benda, 1958) and during co-feeding of ticks on the same host (Labuda et al., 1993; Labuda et al., 1997).

I. trianguliceps, *I. hexagonus* and *I. arboricola* are considered as amplifying vector ticks of TBEV (Grešíková, 1972, Křivanec et al., 1988). Infected secondary vector ticks, such as *Haemaphysalis inermis* and *Dermacentor reticulatus* exhibit lower transmission rates (Grešíková and Kaluzová, 1997). The virus was also isolated from *H. inermis* in the Czech Republic (Grešíková and Nosek, 1966), and *H. spinigera* and *H. turturis* were shown to be able to transmit TBEV under laboratory conditions (Nosek et al., 1967). Kožuch and Nosek (1971) confirmed *D. marginatus* and *D. reticulatus* as possible TBEV vectors, though the infection and transmission rates were lower than in vector species of the genera *Ixodes* and *Haemaphysalis* (Nosek et al., 1967).

Far Eastern and Siberian subtypes are transmitted predominantly by *I. persulcatus*. This tick comprises 80-97% of all tick species in the Urals, Siberia and the Far East region of Russia. *Dermacentor pictus*, *Dermacentor silvarum* and *Hyalomma concinna* have also been associated with local TBE outbreaks in some areas of Siberia and the Far East, where *I. persulcatus* is not the predominant species (Zlobin and Gorin, 1996). However, TBEV has

Table 1. Summary of natural vertebrate host experimentally infected with TBEV

Experimental host	Latin name	Route of inoculation	Signs of infection	Reference
wood mice	*Apodemus sylvaticus*	sc	viremia	reviewed in Karabatsos, 1989
great dormouse	*Glis glis*	sc	viremia (up to the 7th day p.i.)	Nosek et al., 1963
harvest mice	*Micromys minutus*	sc	viremia	reviewed in Karabatsos, 1989
moles	*Talpa europaea*	sc	viremia (2nd-10th day p.i.)	Kožuch et al., 1966
bats	*Myotis myotis*	sc	viremia (high and long lasting)	Kolman et al., 1960
hedgehog	*Erinaceus europaeus*	sc	viremia (long lasting)	Kožuch et al., 1967
European hares	*Lepus europaeus*	sc	viremia	reviewed in Karabatsos, 1989
chickens	*Gallus gallus*	sc	viremia	reviewed in Karabatsos, 1989
domestic ducks	*Anas sp.*	sc	viremia	reviewed in Karabatsos, 1989
passerine	*Passer domesticus*	sc	viremia	reviewed in Karabatsos, 1989
birds	various	sc	viremia/absence of viremia	reviewed in Karabatsos, 1989
lizards	*Lacerta viridis*	sc	viremia, formation of antibodies only at temperature >30°C occasional clinical signs of encephalitis	Grešíková-Kohútová and Albrecht, 1959.
roe deer	*Capreolus capreolus*	sc	low level of viremia	Libíková and Albrecht, 1959
cows	*Bos primigenius* f. *taurus*	sc	viremia, excretion of the virus into the milk	Grešíková et al., 1957
dogs (puppies)	*Canis lupus* f. *familiaris*	sc	low level of viremia, antibody formation	Grešíková et al., 1972

been isolated also from 15 other tick species in Russia, but sporadically also from other parasitic invertebrates, e.g. fly, flea and lice (reviewed in Gritsun et al., 2003b).

I.3.8 Natural vertebrate hosts

Hosts of TBEV in nature can be divided into reservoirs, indicators and accidental hosts. Reservoir host are capable of transmitting infection. This capability is referred to as 'reservoir competence'. These animals are sensitive to the virus able to multiply the virus, including viremia for a long period of time with high virus titer without becoming clinically ill. Indicator hosts have only brief viremia with low virus production and are not able to transmit the virus to vectors. Accidental hosts can develop viremia, but they do not participate in virus circulation and develop clinically apparent or inapparent infection (reviewed in Süss, 2003).

Usually, reservoir hosts belong to the groups of Rodentia (*Clethrionomys*, *Apodemus*, *Mus*, *Microtus*, *Micromys*, *Pitymys*, *Arvicola*, *Glis*, *Sciurus* and *Citellus*) (Kožuch et al., 1967), Insectivora (*Sorex*, *Talpa*, *Erinaceus*) (Kožuch et al., 1967) and Carnivora (*Vulpes*, *Mustela*). Indicator hosts are e.g. foxes (*Vulpes*), badgers (*Meles*), weasels (*Mustela*) (Radda et al., 1969), genera of Chiroptera (*Myotis*, *Plecotus*, *Rhinolophus*, *Barbastella*) (Kolman et al., 1960), of Aves (*Phasanus*, *Perdix* and other genera which live mainly on the ground), Duplicenta (*Lepus*), and Artiodactyla (*Capreolus*, *Cervus*, *Rupicarpa*, *Sus*, *Alces*, *Bos*, *Ovis*, *Capra*) (Libíková and Albrecht, 1959) (reviewed in Karabatsos, 1989; Süss, 2003).

Animals that were infected with TBEV under laboratory conditions as experimental hosts are summarized in **Table 1**.

I.3.9 Routes of transmission

Transmission cycles are determined by the interactions between tick-borne viruses, their vectors and their vertebrate hosts. This is a triangle on three levels: pathogen–host interactions, pathogen–vector interactions, and host–vector interactions (reviewed in Süss, 2003).

In general, humans are infected with TBEV following the bite of an infected tick when walking through dense vegetation in forests. The virus is transmitted by saliva during first minutes of feeding. Interestingly, the titer of the virus increases by 10-100 times during the first to the third day of feeding. Number of pharmacologically active compounds is secreted in tick saliva. These modify the microenvironment in the site of feeding and control the haemostatic, inflammatory and immune responses in the vertebrate host in order to facilitate blood feeding. Such bioactive saliva molecules include immunoglobulin-binding proteins, histamine-binding proteins, interferon regulators, and natural killer cells and complement inhibitors. The action of the bioactive saliva molecules facilitates tick-borne pathogen transmission to the vertebrate host (reviewed in Nuttall and Labuda, 2004).

Serological surveys suggest that between 70 and 95% of human infections in endemic regions are sub-clinical (asymptomatic) (Kopecký et al., 1991; Luňácková et al., 2003; Gritsun et al., 2003b). Thus, TBEV appears to infect high proportion of humans relatively harmlessly.

Another natural route of human TBEV infection is associated with the consumption of non-pasteurized goat, sheep and cow milk and the virus remains stabile for relative long period also in various milk products such as yoghurt, cheese and butter (Grešíková, 1959b). A particularly unusual outbreak was caused by infected goat milk in the Rožňava district of Slovakia in 1951-52, when at least 660 people were infected (Blaškovič, 1954). The latest relatively small outbreak of TBE by alimentary route was reported in 1999 in the Czech Republic. In this case, 22 people were infected by consumption of sheep cheese. Some of the cases were severe (Daneš, 2000). In 2005 and 2007, seven people in total were infected by consumption of goat milk.

Experimentally infected goats, sheep and cows secreted the virus to the milk, however, there were differences in the duration of the secretion and the titer of the virus in the milk (Málková, 1960). Cows secreted the virus to the milk for up to 6 days p.i., but the titer was significantly lower when compared with goats and sheep (Grešíková, 1957, 1958; Grešíková and Řeháček, 1959). On the contrary, TBEV can be isolated from the milk of goats for 5–25 days following infection (reviewed in Gritsun et al., 2003b). With the aim to decrease the risk of TBE infection of men by alimentary route, a life attenuated TBEV vaccine for goats was developed (Mayer, 1966). However, recent molecular analysis of the vaccine strain revealed that this strain is not an attenuated variant of TBEV, but a strain of virus Langat, possibly a result of laboratory contamination of cell cultures (Růžek et al., 2006).

TBEV is stable for up to two hours in normal gastric juice at pH 1.49–1.80 and in gastric juice with reduced acidity (pH 1.87–2.21). In gastric juice taken from humans after a meal (pH 2–7) the virus infectivity is stable for 2 hours (Grešíková, 1959a). Milk move out of the stomach quite quickly (the first milk consumed reaches the duodenum within minutes and after 1.5–2 h there is no milk in the stomach) (Gritsun et al., 2003b).

Cases of laboratory TBEV infections have apart from needle-stick injuries also been associated with aerosol infection of laboratory personnel. In the prevaccination era, these laboratory-acquired infections were not rare (Gallia et al., 1949; Molnár and Fornosi, 1952; Hoffmann, 1973; Bodemann et al., 1977; Avšič-Županc et al., 1995).

I.3.10 Pathogenesis of TBE

The pathogenesis of TBE remains in many areas of interest unresolved. However, the general features of the pathogenesis are similar to other tick-borne viruses within the family *Flaviviridae* (**Fig. 4**). After the tick bite, the virus replicates in subcutaneous tissues (Labuda et

al., 1996). Dendritic cells in the skin are likely to serve as a vehicle for the transport of the virus to draining lymph nodes (reviewed in Chambers and Diamond, 2003). The lymph nodes play an important part in the pathogenesis of TBE, although virus replication is not accompanied by virus-specific histological changes in the nodes. Moreover, no marked destruction of infected lymph node cells was observed (Málková and Filip, 1968). Massive viral multiplication in the nodes leads to the spreading of virus into the blood stream and induction of viremia (Málková and Fraňková, 1959; Málková and Kolman, 1964; Málková et al., 1969). Temporary leukopenia in the white blood picture is observed. A significant decrease is recorded in all cellular elements. In regional lymph nodes, a significant decrease in lymphocytes appears (Málková et al., 1961).

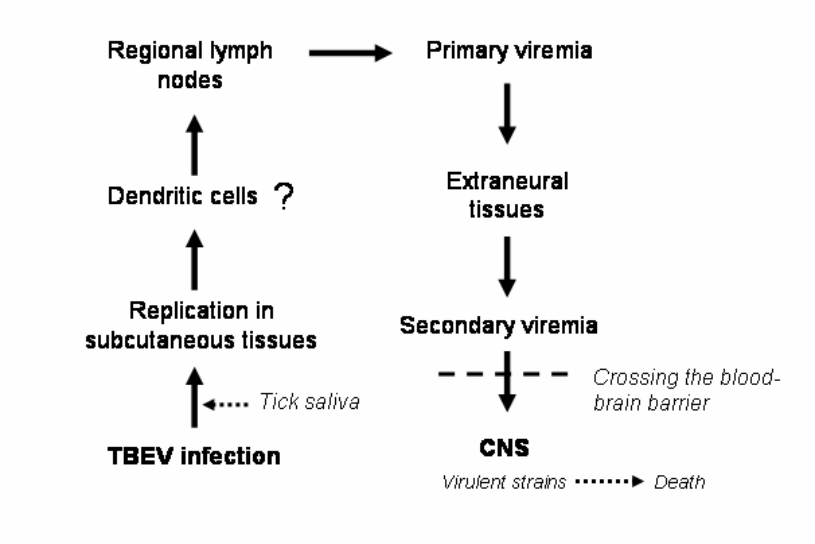

Fig. 4. General scheme to explain the mechanism of pathogenesis of TBE in subcutaneously inoculated laboratory mouse.

Many extraneural tissues are infected during the viremic phase and, subsequently during the secondary viremia, the virus invades the central nervous system. The ability of viruses to invade the central nervous system after the replication in extraneural tissues and induction viremia is referred to as 'neuroinvasiveness' (McMinn, 1997), and represents a critical step in the pathogenesis of the flavivirus encephalitis. Otherwise, the ability of virus to initiate cytopathic infection in the brain and to cause encephalitis is referred to as 'neurovirulence' (McMinn, 1997).

Characteristic, but not disease-specific neuropathologic changes in CNS that include meningitis and polioencephalomyelitis accentuated in spinal cord, brainstem and cerrebellum associated with inflammatory cell infiltration were described in mice (Osetowska and Wróblewska-Mularczyk, 1966), hamsters (Simon et al., 1966), monkeys (Simon et al., 1967), as well as in humans (Seitelberger et al., 1966; Környey, 1978; Beer et al., 1999; Schellinger et al., 2000, Gelpi et al., 2006a,b). Although TBEV induces apoptosis in human neuroblastoma and glioblastoma cell lines (Růžek et al., manuscript in prep.; **Chapter II.6**) and also in mouse and monkey brain neurons (Isaeva et al., 1998; Kamalov et al., 1998), prominent signs of neuronal apoptosis were not seen in postmortem brain tissues from human TBE patients, as demonstrated by anti-caspase 3 immunohistochemistry and TUNEL assay (Gelpi et al., 2006b). On the other hand, prominent inflammatory infiltrates and cytotoxic T-cells were observed in close contact with morphologically intact neurons suggesting a key role for cytotoxic T-cells in the development of encephalitis (Gelpi et al., 2006a,b). These data are supported by the results on the transfer of sensitized splenocytes to immunosuppressed mice, challenged with a Far Eastern strain of TBEV. Transferred splenocytes shortened the incubation period of the disease implying a pathogenic role for the immune system in TBE (Semenov et al., 1975). In addition, TBEV was also isolated from the brains of healthy animals in wild and Syrian hamsters in the laboratory indicating that propagation of TBEV in brain tissue is not necessarily accomplished by apoptosis (reviewed in Gritsun et al., 2003a).

Experiments with several mice breeds that included knockouts in $CD8^+$ and/or $CD4^+$ genes, and adoptive transfer of specific cell populations to SCID mice revealed that $CD4^+$ T-cells may have some function in the control of TBE, whereas $CD8^+$ T-cells play a role in immunopathology of TBE caused by neurovirulent strains. However, in the absence of immune response the virus on its own is also capable to cause encephalitis (Růžek et al., 2008 submitted; Chapter II.5).

Although the pathogenesis of TBE has been investigated extensively, the mechanism by which the virus breaches the blood-brain barrier and is transported across the cerebral blood vessels and mechanisms of spread of TBEV within the brain remains ambiguous. In case of visualization of TBE virus in brain sections of fatal human cases, the virus was detected in neuronal processes and perikarya, suggesting the selective tropism of the virus to neurons (Gelpi et al., 2005). However, the virus was not detected in glial cells (Gelpi et al., 2005), in contrast to previous studies (Mázló and Szántó, 1978). The virus was most considerably found in anterior horns, medulla oblongata, pons, cerebellum and striatum (Gelbi et al., 2005).

The possible mechanisms for entry of TBEV through the blood-brain barrier are e.g. (i) passive diffusion, (ii) transcytosis, or spread via (iii) the olfactory nerve (Haglund and Günther, 2003).

In the study by Jandásek (1958a), mice had been injected with TBEV (strain Hypr) peritoneally and the penetration of human serum, penicillin and trypan blue into the brain was investigated. At any time points post infection, the human serum could not be found in the brains by means of precipitation. Traces of the penicillin could be found in the brain at the first, second and third day p.i. and especially during the terminal phase of the infection. Trypan blue was not observed in the brain parenchyma at any time p.i. However, trypan blue proved to be more toxic for the infected mice than for healthy ones (Jandásek, 1958a).

In other study by Jandásek (1958b), cornea and vitreous body of mouse eyes have been subjected to inoculation with TBEV, strain Hypr. The aim of the study was to find whether the virus penetrates from the periphery into the brain by way of blood or nerves. The method employed brought indirect information. The mice with inoculated cornea or vitreous body are not very susceptible to infection in this way. Scarification of the cornea did not increase the susceptibility of the inoculated animals. Subsequently to the inoculation of the cornea, viremia was observed in almost all animals. Viremia appeared most frequently on the third day p.i. that corresponds to the viremia, which can be observed after small inocula given subcutaneously or intraperitoneally. It seems that the virus inoculated on the cornea is absorbed into the blood circulation probably though the conjunctiva. Therefore, the route by way of nerves is not very probable (Jandásek, 1958b).

Mice inoculated intranasally (i.n.) with TBEV develop fatal encephalitis. However, the mean surviving time of the mice is analogous to mice inoculated subcutaneously (s.c.). The i.n. infected mice develop viremia, as in the case of s.c. or intraperitoneal inoculation (Růžek et al., unpublished results).

I.3.11 Molecular determinants of virulence

Noticeable differences in neurovirulence and neuroinvasiveness were observed in different strains of TBE. Different virulence was observed also during trials on laboratory animals (Gritsun et al., 2003b).

Great number of differences on the level of amino acid sequence was detected in whole-genome sequence of some strains of TBEV (Wallner et al., 1996) as well as in partial sequences encoding viral proteins.

In general, mutations especially in the E protein are supposed to influence the virulence of TBEV. E protein is the main antigenic determinant and is responsible also for viral entry to the host cell. Amino acid substitutions in E protein cause decrease in neuroinvasiveness, although neurovirulence is usually not reduced (McMinn, 1997). The highest number of attenuating

mutations in E protein was revealed in domain probably binding to specific cell receptor and participating in membrane fusion (Holzmann et al., 1995). Mutants, escaping neutralizing effect of monoclonal antibodies, contained amino acid substitutions in E protein (Holzmann et al., 1997).
A comparative study of sequences coding structural protein E of strains causing severe hemorrhagic syndrome with other strains of TBEV revealed 13 unique amino acid substitutions. These substitutions can be found also in other representatives of *Flaviviridae* including ones also causing hemorrhagic fever. These findings support the importance of E protein for the course of the disease (Ternovoi et al., 2003).
Adaptation of TBE virus (strain Neudoerfl) to cell line BHK-21 (baby hamster kidney) led to a number of mutations in E protein, that increased the virus affinity to cells expressing heparan-sulfate receptors on their surface in comparison with the original strain (Kroschewski et al., 2003). In the course of 16 independent experiments, 12 different protein E mutation patterns were identified. These were located in all three of the structural domains and distributed over almost the entire upper and lateral surface of protein E. The mutations resulted in the formation of local patches of predominantly positive surface charge. Recombinant viruses carrying some of these mutations in a defined genetic backbone showed heparan sulfate (HS)-dependent phenotypes, resulting in an increased specific infectivity and binding affinity for BHK-21 cells, small plaque formation in porcine kidney cells, and significant attenuation of neuroinvasiveness in adult mice (Mandl et al., 2001).
Similarly, the strain Oshima 5-10 from Far East was adapted to cell line BHK-21. Mutants had markedly decreased neuroinvasiveness but unchanged neurovirulence. Sequence analysis identified two amino acid substitutions localized in sequences coding for proteins E and NS5. Also in this case, the amino acid substitution in E protein led to its higher affinity to glycosaminoglycans (Goto et al., 2003).
Molecular basis of virulence was further studied by construction of virus mutants, carrying genetic mutations throughout the genome. Differences in biological characteristics between original and mutant strains were subsequently investigated. The phenotype of mutants with deletion in variable (proximal) region of 3´ noncoding region was not changed. Deletions in highly conserved 3´ distal element resulted in considerable attenuation for laboratory mouse, although growth characteristic of virus in tissue culture was not altered (Mandl et al., 1998).
Deletion in sequence coding for capsid protein C also resulted in marked attenuation of TBEV. Deletions with extent to 17 % of mature C protein still had no effect on virus replication in host cell. These viral mutants were highly immunogenic for laboratory mice (Kofler et al., 2002).
The viral protease (NS2B-NS3) is believed to be an important determinant of virulence/attenuation of different flaviviruses (Yellow fever, dengue, West Nile virus).
Moreover, the importance of these proteins in virulence of natural isolates of TBE virus was demonstrated when two mutations in NS2B and NS3 genes affected neuroinvasiveness for laboratory mouse and led to the temperature-sensitive phenotype of the virus (Růžek et al., 2008b).
In previous studies, it was established that the NS3 cleavage activity is necessary for correct processing of the viral polyprotein (Chambers et al., 1990b). It was suggested that the cofactor activity of NS2B is localized in the central hydrophilic region flanked with hydrophobic parts (Chambers et al. 1993, reviewed in Lindenbach and Rice, 2003). The catalytic site of NS2B-NS3 complex involves a conserved central region of NS2B and N-terminal region of NS3.
NS3 is the second largest nonstructural protein with several enzyme activities and it is highly conserved among flaviviruses. The N-terminal 180 amino acids mediate the protease activity of NS2B-NS3 (Chambers et al., 1990b) and the C-terminal region is implicated in RNA replication (helicase and RNA triphosphatase activities). Mutations introduced into the NS3 catalytic site (Ser, 138) of TBE virus abolished self-cleavage between NS2B and NS3 proteins

when the NS2B-NS3 part of the viral genome was expressed in vitro. Analogous results were obtained for other flaviviruses, e.g. West Nile virus (Wengler et al., 1991).
TBE virus can be attenuated also by inserting of a specific combination of point mutations; each of them contributes to resulting reduction of virulence. These point mutations can be localized in sequences coding for structural and nonstructural proteins as well as in 3' noncoding region of viral genome (Gritsun et al., 2001).

I.3.12 Models in TBEV research

I.3.12.1 Cell cultures

TBEV grows in a number of mammalian as well as invertebrate cell lines. For example, cell lines Detroit 6 (von Zeipel and Svedmyr, 1958), porcine kidney stable (PS) cells (Korych, 1960; De Madrid and Porterfield, 1969, Kožuch and Mayer, 1975), HeLa (Libíková and Albrecht, 1961; Libíková and Vilček, 1961) and monkey kidney cells CV-1 (Hronovský et al., 1978) were used for virus cultivation. Plaque assay is usually performed on PS cell monolayers (De Madrid and Porterfield, 1969).
Human neural cells (neuroblastoma, glioblastoma and medulloblastoma) and mouse neuroblastoma cells were shown to be very sensitive to TBEV, and the virus replication leads to very high titers (Růžek et al., in prep.; Chapter II.6). The character of cytopathic effect (CPE) varied according to the virulence of the virus, as demonstrated by the comparison of CPE caused by TBEV strains Neudoerfl (mildly virulent) and Hypr (highly virulent).
An investigation of the relationship between TBEV and ticks (interaction of the virus with the cellular receptor, multiplication and the maturation process of the virus in the tick cells) necessitates the use of tick cell lines. First attempt to infect tick cells with TBEV was performed by Řeháček (1962) in the primary culture derived from *Dermacentor marginatus* ticks (Řeháček, 1958; 1962). It was observed that the virus multiplied in the tick cells without causing any CPE.
Moreover, the growth of TBEV was also investigated in lines derived from vector *Ixodes ricinus* ticks (IRE/CTVM18, 19, and 20), as well as various non-vector ticks, namely *Ixodes scapularis* (IDE2), *Boophilus microplus* (BME/CTVM2), *Hyalomma anatolicum anatolicum* (HAE/CTVM9), *Rhipicephalus appendiculatus* (RA-257) and lines from the argasid tick *Ornithodoros moubata* (OME/CTVM21 and 22). All the tick cell lines tested were susceptible to infection by TBEV and the virus caused productive infection without any cytopathic effect. However, there was a clear difference between the TBEV growth in vector and non-vector cell lines, since *I. ricinus* cell lines produced 100-1,000-fold higher virus yield than the non-vector cell lines. The lowest virus production was observed in *O. moubata* and *R. appendiculatus* cell lines (Růžek et al., 2008a; Chapter II.3).
The C6/36 mosquito cells are not sensitive to the infection with TBEV (Lawrie et al., 2004).

I.3.12.2 TBEV and laboratory animals

Most of the experiments with TBEV under in vivo conditions (summarised in **Table 2**) were performed using rodents. Rodent models continue to be relied upon for studies of general pathogenic processes as well as of viral neurovirulence determinants or immune system requirements for a successful antiviral response (reviewed in Chambers and Diamond, 2003; Nalca et al., 2003). In fact, research on flaviviruses introduced laboratory mice as animal models in virology, when it was reported in 1930 that newborn mice inoculated intraperitoneally with a flavivirus (yellow fever virus) died of encephalitis.
The most practical and frequently used rodents in TBEV research are laboratory mice, such as strains Balb/c, Swiss Albino mice (Hoffmann and Radda, 1968) or C57Bl/8 (reviewed in Mandl, 2005). In contrast to wild rodents, laboratory mice are susceptible to TBEV infection

and develop lethal infection of CNS (Simon et al., 1966). The symptoms of the infection are analogous to severe cases of TBE in humans.
Suckling or juvenile mice are the most sensitive experimental animals to TBEV infection. The infection is lethal after both intracranial as well as peripheral inoculation.
TBEV multiplies also in brains of suckling white rats and suckling Syrian hamsters.
Adult Syrian hamsters were shown to react with inapparent infection after peripheral TBEV inoclulation. The presence of the virus in CNS was observed in 20% of TBEV infected, clinically normal hamsters. However, after subcutaneous infection with 10^3 intracerebral mouse LD_{50} of the virus, hamsters exhibit 25% mortality (Slonim and Závadová, 1977a).
Guinea pigs and rabbits exhibit inapparent infection after the inoculation with TBEV.
TBEV can be also inoculated into yolk sack of chicken embryos. The embryos die 4-7 days post inoculation (Slonim, 1956).
TBEV also causes meningoencephalitis in monkeys *Maccaca mulatta* and *M. cynomolga* after intracerebral or intraspinal inoculation. Subcutaneous inoculation of monkeys leads to a clinically inapparent form of infection with viremia, antibody formation (Slonim and Závadová, 1977b), but there are no characteristic histological changes in the CNS (Benda et al., 1960, Slonim et al., 1966, Mayer et al., 1968).
Different experimental parameters have been used to compare pathogenic properties among TBEV strains. These include e.g. mean survival time, mortality, 50% lethal dose (LD_{50}), and 50% infectious dose. 'Neurovirulence' is tested after intracranial inoculation of the virus into laboratory animals. On the other hand, 'neuroinvasiveness' is examined after peripheral (subcutaneous or intraperitoneal) inoculation.

Table 2 Summary of laboratory animals used in TBEV research (reviewed in Karabatsos, 1989)

Experimental host	age	inoculation route	signs of infection
mice	newborn	ic	fatal encephalitis
		ip	fatal encephalitis
	adult	ic	fatal encephalitis
		ip	fatal encephalitis
		sc	fatal encephalitis
white rats	2 days	ic	fatal encephalitis
	11 days	ic	fatal encephalitis
	30 days	ic	fatal encephalitis
Syrian hamsters	young	ic	fatal encephalitis
	adult	sc	inapparent infection
rhesus monkeys		ic (after adaptation)	fatal encephalitis
cynomolgus monkeys		in	viremia, antibody formation
		sc	viremia, antibody formation
goat, sheep	young	ic	clinical signs of encephalitis
	adult	sc	viremia, excretion of the virus into the milk, antibody formation
swine		sc	antibody formation
embryonated egg			virus multiplication

I.4 Specific aims

1. Study molecular epidemiology of TBEV in the Czech Republic in time period 1948-2002.
2. Develop a simple and rapid method for subtyping of TBEV-isolates.
3. Study the interaction of TBEV and cells derived from various vector and non-vector ticks.
4. Study the microevolution of field TBEV isolate with the purpose to identify potential molecular determinats of virulence of TBEV.
5. Study the role of host immune system in the pathogenesis of TBE.
6. Study interaction of TBEV with human neural cells.

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Chapter II.1

Molecular epidemiology of tick-borne encephalitis virus in Central Europe (since its discovery up to the present, 1948-2002).

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MOLECULAR EPIDEMIOLOGY OF TICK-BORNE ENCEPHALITIS VIRUS IN CENTRAL EUROPE (SINCE ITS DISCOVERY UP TO THE PRESENT, 1948-2002).

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Key words: tick-borne encephalitis virus, molecular epidemiology, envelope protein, non-coding region

Abstract

We performed an extensive molecular epidemiological analysis of tick-borne encephalitis virus strains isolated in the Czech Republic since the discovery of the virus in Europe in 1948 up to the present, to determine the structure of viral genetic diversity in the region of Central Europe and the evolutionary processes occurring within the genome of the virus.
We analyzed molecular characteristics of strains from different time periods and different sources that included human samples, ticks and natural hosts. The molecular analysis was based on the complete sequence analysis of envelope (E) protein gene and 3' non-coding region. The study revealed minor genetic variations of the virus within the time period of more than 50 years, exhibiting a maximum of only 1.3% at the amino acid level. However, amino acid substitutions identified in the archival strains were not distributed randomly within the sequence of the protein E, but most of them were clustering into two areas of mutations affecting virulence of flaviviruses, including the potential receptor-binding domain. Phylogenetic analysis revealed several distinct groups with high bootstrap support, corresponding to the time period of the isolation and some groups being in correlation with the epidemic activity of the virus. In contrast to previously published studies, three conservative and one semi-conservative deletion was identified within the 3'UTR, without any association with the source of isolation and number of passages in laboratory. The uniformity of deletions within 3'UTR suggests its importance for the properties of the virus under natural ecological conditions.

Introduction

Tick-borne encephalitis (TBE) is the most important arbovirus neuroinfection in Europe and Asia (Gritsun et al., 2003). It is caused by *Tick-borne encephalitis virus* (TBEV), a member of the genus *Flavivirus* within the family *Flaviviridae* (Thiel et al., 2005).
The tick-borne flavivirus (TBFV) group is subdivided into the Mammalian and Seabird virus group, depending on the association of virus with mammalian or seabird ticks, and TBEV comprises of the several virus species within the Mammalian group, previously called TBEV–serocomplex. The TBEV and also other viruses within the Mammalian group are transmitted in nature by ticks; they are widely spread across the Northern Hemisphere, in correspondence with the location of forested areas suitable for the tick habitats, from the Far East Russia to the

Atlantic Ocean. Phylogenetical analysis established that they form so called cline that illustrates gradual change the TBFV virus genomes during their adaptation to the new hosts, i.e. tick and rodent species. On the basis of serological relationships, all the TBEV strains were subdivided into the Far Eastern, Siberian and European subtypes (FE, SIB and E); this classification was also correlated with their geographical distribution and later confirmed by the phylogenetical analysis (Ecker et al., 1999).

In the Czech Republic (formerly Czechoslovakia), the incidence of TBE is the highest in Europe, with 400-800 admitted cases annually. In 2006, 1,029 human cases of TBE were reported in the Czech Republic, which represents the highest incidence ever recorded, indicating significantly increased epidemic activity of the virus in the area of Central Europe (Daniel et al., 2008).

Although the first hints of the existence of tick-borne encephalitis date back to the 18th century, the first medical description of the disease was given by Austrian physician H. Schneider in 1931 (Schneider, 1931). In Europe, tick-borne encephalitis virus was first isolated from human patients in the region of the Czech Republic in 1948 (Krejčí, 1949b; Gallia et al., 1949) when the incidence of clinical manifestations caused by the virus was so high, that it was noticed by infectologists in affected regions (Krejčí, 1949a). Simultaneously, the virus was isolated also from ticks *Ixodes ricinus* suggesting the role of the tick as a vector of the disease (Rampas and Gallia, 1949).

TBE virus is a spherical enveloped virus with a diameter of approximately 50 nm (Slávik et al., 1970). It has an icosahedral capsid containing a single-stranded RNA genome of positive polarity with approximately 11,000 nucleotides in length. Unlike the majority of animal (+)RNA viruses, the 3' terminus does not contain the poly(A) sequence. This part of genome forms a hairpin loop and contains several conserved regions. The length of 3' non-coding region of different TBEV strains is somewhat irregular (in the range of 450 to 800 nucleotides) (Proutski et al., 1997; Gritsun et al., 1997). The viral RNA possesses a single open reading frame coding a polyprotein of approximately 3,400 amino acids. This is co-translationally and post-translationally cleaved by viral and cellular proteases into three structural proteins (C, M, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Chambers et al., 1990; Lindenbach and Rice, 2003). Glycoprotein E, the main component of the virus surface, mediates receptor binding and fusion activity after uptake by a receptor mediated endocytosis. This protein is the main target of neutralizing antibodies and induces protective immunity in the infected or immunized organisms. For its functional importance, it is believed that the E protein is also an important determinant of virulence (Gritsun et al., 1995). Three-dimensional structure of the E protein has been determined by X-ray crystallography (Rey et al., 1995).

In general, RNA viruses, including flaviviruses, exhibit high mutation frequencies. Continuous production of mutants favors adaptability of the virus in the event of environmental changes. However, factors affecting genetic stability of RNA viruses and affecting its rapid change remain mostly unresolved (Domingo et al., 1997). Tick-borne encephalitis virus seems, interestingly, to be remarkably genetically stable under natural ecological conditions. Almost no variability with respect to the pattern of intracellular proteins was found when isolates from different European countries were compared (Heinz and Kunz, 1981; 1982). Similarly, peptide mapping as well as the analysis of the antigenic structure of the E protein by the use of monoclonal antibodies defining different epitopes of the E protein of the strains isolated in Austria with the strains from the same foci isolated 14 years earlier did not provide evidence for any antigenic variation (Guirakhoo et al., 1987). Comparative sequence analysis of the strains isolated in endemic areas of Europe and Asia also revealed low degree of variation between strains within subtypes, reaching a maximum of only 2.2% at the amino acid level (Ecker et al., 1999).

In order to get more information on the evolution and variability of TBE virus, we have determined sequences of the E protein gene and 3' UTR of 34 virus strains isolated in the Czech Republic in a time period since the virus-discovery in 1948 up to the year 2002 and have analyzed their phylogenetic relationships. Determined amino acid substitutions in the E protein of the analyzed strains were superimposed on the 3D structural model of the protein and discussed with the respect to the antigenic and virulence variations.

Material and Methods

Virus strains

Table 1 summarizes the TBEV strains that were analyzed in this study. The strains were isolated from human patients, ticks or natural hosts in the Czech Republic over a period of more than 50 years, since the first discovery of TBEV in Europe up to the present time. The isolates were propagated through suckling mouse brain passage; in majority, low-passage strains were used in this study (Table 1). Stocks of the viruses were prepared as 20% (w/v) mouse brain homogenates and were stored as freeze-dried suspensions.
The analyzed strains were compared with other previously sequenced strains from the region of Central Europe (Table 2).

Viral RNA isolation, RT-PCR and sequence analysis

Viral RNA was isolated from 20% (w/v) mouse-brain suspension using a commercial kit (Qiagen QIAamp Viral RNA Kit, Qiagen) according to the recommendations of the manufacturer.
The cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the instructions for the synthesis of first strand cDNA suitable for PCR amplifications, with 1 μl of an appropriate antisense primer (0.5 μM). All primers used in this study were designed using Vector NTI Suite 5.5.
Nucleotides from positions 963 to 2,491 (E gene) and 9,792 to 11,141 (3' UTR; Neudoerfl strain numeration) were amplified using PCR. 1 μL of RT reaction mixture was subsequently used as a template for PCR amplification of specific genomic regions. PCR amplification was done using the Plain PPP Master Mix (Top-Bio, Czech Republic; 2x; 150 mM Tris-HCl, pH 8.8, 40 mM $(NH_4)SO_4$, 0.02% Tween 20, 5 mM $MgCl_2$, 400 μM dNTPs, 100 U/ml Taq DNA polymerase); 1 μL of each primer (stock 0.01 mM), PCR water up to 25 μL of reaction volume. The cycling conditions were as follows: 5 min of denaturation at 95 °C, followed by 30 cycles with 95 °C for 30 s, 55 °C (for amplification of E gene) or 47°C (for amplification of 3' UTR) for 30 s and 72 °C for 1 min 30 s. The resulting PCR products were separated by 1.7 % agarose gel electrophoresis, visualized by UV light, cut out of the gel and the DNA was eluted using the QIAquick Gel Extraction kit (Qiagen).
Nucleic acid sequences were determined by direct sequencing of both strands of PCR products. The cycle sequencing reactions were performed using a CEQ™ Dye Terminator Cycle Sequencing Kit (Beckman Coulter). 4 μL of the purified PCR product was mixed with 1 pmol primer, 9 μL water and a reaction mixture containing the four-labeled dideoxynucleotide terminators. The sequences were determined with the automated Beckman CEQ 2000 DNA Analysis System (Beckman Coulter).
The nucleotide and deduced amino acid sequences were analyzed using BioEdit Sequence Alignment Editor, version 5.0.6. (http://www.mbio.nscu.edu/BioEdit/bioedit.html) and GeneDoc, version 2.6.002 (http://www.psc.edu/biomed/genedoc) and aligned with other available genomic sequences of TBEV strains. Full alignments are available on request.

Data analysis

To determine the evolution and genetic relatedness among TBEV isolates, phylogenetic trees were constructed from the aligned nucleic acid sequences. Phylogenetic trees were estimated by Neighbor-Joining method using MEGA-Molecular Evolutionary Genetics Analysis, version 4.0 (http://www.megasoftware.net). To determine the support for different nodes on the tree, we conducted a bootstrap analysis using 1000 replications.
Statistical parsimony analysis revealing genealogical relationships of all sequenced strains and other previously sequenced TBEV strains isolated in Central Europe was performed using TCS software package (Clement et al., 2000).

3D structure analysis of E protein
The predicted three-dimensional structure of the envelope glycoprotein (1SVB.pdb) and the superimposition of mutations were produced using PYMOL facilities available at http://pymol.sourceforge.net/ and the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen et al., 2004).

Analysis of selection pressure
To determine the nature of the selection pressure on TBEV, an analysis was made of the ratio of non-synonymous (d_N) and synonymous (d_S) nucleotide substitutions per site. Positive selection on TBEV gene E was indicated by $d_N > d_S$. Estimates of average codon-based evolutionary divergence over all pairs of obtained sequences of E gene were based on the pairwise analysis conducted using the modified Nei-Gojobori (assumed transition/transversion bias = 2) method in MEGA4 (Zhang et al., 1998; Tamura et al., 2007).

3' UTR secondary structure prediction
The predicted secondary structures of the 3' UTR were produced using Mfold server, version 3.1 (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi). The method is based on the algorithm predicting a minimum free energy, as well as minimum free energies for foldings that must contain any particular base pair, and therefore allow the finding of the optimal and suboptimal secondary structures of single stranded RNA molecules (Zuker, 2003).

Results
Thirty-four TBEV strains isolated in the Czech Republic in the time period 1948 to 2002 were analyzed with respect to the sequences of E gene and 3' UTR. RNA isolated from each TBEV isolate was subjected to RT-PCR using the primers and procedures described in Material and Methods. PCR products were purified and sequenced directly.

Comparison of the deduced amino acid sequences of the E protein
On the basis of the comparison of the deduced amino acid sequences of the E protein of TBEV isolates revealed minor sequence variation occurring in one geographic region in the time period 1948 up to the year 2002. The amino acid identities of the E protein sequences between the studied strains were 98.7-100%; overall identities of the strains within the European subtype ranged from 97.9 to 100% in correspondence with other data explaining the slow evolution of TBEV due to the slow life cycle of the ticks (Gritsun et al., 1995). Identified amino acid substitutions are summarized in Table 3.
Amino acid variations were found at 25 positions; only 8 were present in more than one strain. In some cases, similar substitutions were common for the isolates from the same time periods (e.g. strains Riha and Petracova; 9001, 8641 and 9025). Interestingly, previously reported amino acid substitutions of Hypr strain, with the exception of the substitution at the subtype-signature position 437 (Wallner et al., 1996), were not confirmed by this study. At these

positions, Hypr strain carried wild type substitutions, when low- as well as high-passaged variants were analyzed.
We found amino acid substitutions at positions previously described as molecular determinants of flavivirus virulence or in their close proximity. For example, the positions 52, 68 or 333 were found to be molecular determinants of neuroinvasiveness or neurovirulence in virus Langat, West Nile virus, Japanese encephalitis virus and yellow fever virus (TBEV strain Neudoerfl numeration; Chambers et al., 1998; Schlesinger et al., 1996; Chambers and Nickells, 2001; Hasegawa et al., 1992; Pletnev and Men, 1998).
Substitutions at positions 317 (strain 9045) and 433 (strain Porodova) are of special interest. Far Eastern (FE)-strains carry Ile at the position 317, S-strains Thr, and for European (E)-strains this position is non-conservative. The strain Porodova carried Val at the position 433, amino acid typical for both Siberian (S)- and FE-strains, but not for the E-strains at this position.
Amino acid substitutions identified in the analyzed strains and superimposed in the three-dimensional structure of E protein of strain Neudoerfl (Rey et al., 1995; Fig. 4) seemed to be not distributed randomly in the sequence of E protein. Some substitutions were found to be in close proximity to each other and clustered in specific regions. These substitutions cluster in the domain III of the protein E, which is believed to be a receptor-binding domain. Another region of observed substitutions is in domain I, in cluster D that is believed to be one of the areas of mutations affecting virulence of flaviviruses. Generally, most of the identified substitutions are exposed on the upper or lateral surface and only one substitution is completely buried inside the protein.

Phylogenetic and evolution analysis
The number of synonymous nucleotide changes from the oldest analyzed TBEV isolate (Riha/1948) was shown to accrue linearly with time; however, the number of non-synonymous mutations did not exhibit increasing tendency.
Phylogenetic tree based on the sequences of E gene is presented in Fig. 1. The analysis showed that all studied TBEV isolates belong to the lineage of European subtype strains. Interestingly, isolates, with only a few exceptions, fall into distinct groups in accordance to the time periods of isolation. For example, strains from the period 1954-1967, or 1968 and 1966 fall into one group; similarly isolates from late 70's represent one group associated with the strain Als.I isolated in 1975 in France. Recent isolates (1986-2002), with the exception of isolates from the years 1996 and 1998, grouped in close proximity to the strain Ljub.I., isolated in 1993 in Slovenia. However, also association with place of isolation was observed and therefore, it is not easy to define if year of isolation or the place is of higher importance. No dependence on the source of the isolates that comprised two species of ticks, natural host and human samples was found.
To determine the nature of the selection pressure acting on TBEV E gene, we undertook the analysis of the number of synonymous and non-synonymous differences for all synonymous and non-synonymous sites, respectively. Estimates of average codon-based evolutionary divergences on the basis of synonymous substitutions over all sequence pairs greatly exceeded the values based on non-synonymous differences. The observed average d_N/d_S ratio (corrected per synonymous and non-synonymous site) reached 0.04 for E gene, strongly suggesting the positive selection in TBEV evolution.
Fine-scale statistical parsimony analysis (TCS) shows the evolution of strains clustered in the group of most recent isolates, i.e. from 1986-2002. At a finer resolution, viruses from this group differed by between 1 and 8 nucleotides. Results from this analysis demonstrate clear evolution of the recent strains evolved from the strains isolated in the late 80´s (Fig. 2).

Nucleotide alignment and predicted secondary structures of 3'UTR

For better understanding of the evolution of 3' UTR in TBEV genome, that length has been believed to be without any correlation with a variety of parameters, such as year of isolation, source or geographical origin, we determined and analyzed 3' UTR sequence of 34 TBE virus strains from the period since the discovery of the virus in Europe.

Interestingly, the analysis did not confirm previous data suggesting independent generation of deletions of various lengths. On the contrary, three conservative and one semi-conservative general patterns of the character of the 3'UTR were identified in the analyzed strains that allowed us to divide the strains into four groups (Fig. 5).

(i) Most general pattern (Group 1), that was represented by 67 % of the studied strains, is characterized by lacking only the poly(A) segment typical for Neudoerfl strain. It seems that these strains have full length (i.e. wild type) 3'UTR and that the strain Neudoerfl is rather atypical than typical strain (T.S. Gritsun, personal communication).

(ii) The Group 2 (strains Hypr, 166, 8641, 9001, 9025) contained the largest deletions despite low number of mouse-brain passages. The deletions are not identical but very similar. Also the predicted secondary structures of 3'UTRs are very similar. This group comprises isolates from humans (strain Hypr), tick *I. ricinus* (strains 8641, 9001, 9025) and the hedgehod tick *I. hexagonus* (strain 166). The strains were isolated in 1953, 1977, 1978 and 1986. There is no relation to the years of the isolation of the strains within this group. Very interestingly, the strain Hypr contained identical deletion after 5 passages as well as after >60 passages, as determined in our laboratory (J. Štěrba, unpublished results).

(iii) The strains from human patients from 1948 and 1953 (Riha and Petracova; Group 3) contained virtually identical deletion (from the position 52 to 226 and 53 to 229, respectively). The length of the deletion was independent on the number of passages since strain Riha undergone 54 passages in comparison with 7 passages of the strain Petracova. Moreover, the length of the deletion was shorter when compared with the low-passaged strains from the preceding group. Secondary structure of the 3'UTR region was identical in these two strains.

(iv) Strains 340, 465, 398, 399 and 414 representing Group 4 contained virtually the same deletion (from 119 to ~435 nt; Neudoerfl strain numeration from the start of 3'UTR). All of these strains exhibited also analogous secondary structure of the 3'UTR. Strains 398, 399 and 414 were passaged three times in laboratory mouse, the strains 340 and 465 only once. The strains were isolated in the same region in time period 1954-1967 from natural host as well as from ticks; the strain 465 was isolated from the brain of dead squirrel, while the other strains from ticks *I. ricinus.*

Taken together, 3'UTR exhibited conserved or semi-conserved character with the deletions independent on the source of isolation or number of mouse brain passages with most frequent sequence lacking only poly(A) segment of Neudoerfl strain. Moreover, in some strains, there was an apparent correlation between the year of isolation and the character of deletion in 3'UTR.

Discussion

By studying 34 TBE virus strains isolated in the Czech Republic during the time period 1948-2002, we have provided the largest and most complex molecular epidemiological analysis of this most important arbovirus in the region of Central Europe. This region represents the area with the highest incidence of human cases in Europe. However, the incidence of TBE in humans noticeably varied during the monitored period in this region. The high occurrence of the infection in 1960s gradually decreases and in 1970s and 1980s reached value of 139-400 cases annually. Sporadically, there were more than 400 cases per annum in 1970, 1973, and 1979. A steep increase took place in the 1990s (EpiDat; www.szu.cz), when the annual incidence was more than twofold higher in comparison with preceding period, 400-600 cases

per annum with maximum of 745 cases in 1995 and 706 cases in 2000 (Daniel et al., 2004). A similar increase in number of cases was observed also in other regions of Europe (www.tbe-info.com/epidemiology/). Moreover, clinicians have reported recently occurring changes in the course and seriousness of the clinical manifestation of TBE (V. Chmelík, personal communication). Phylogenetic analysis of the strains from the Czech Republic together with other previously sequenced strains from Central Europe revealed several distinct groups with high bootstrap support, corresponding to the time period of the isolation. For example, group of most recent strains (1986-2002), group of the isolates from the late 70´s or old strains from 1948 and 1966, or 1963-67 are well defined (Fig. 1). The phylogenetic grouping of the isolates relatively correlates with the epidemic activity of the virus in the given time periods. Most obviously, the increase of the incidence since the late 1980´s and the increase of seriousness of the disease can be associated with the emergence of phylogenetically distinct TBEV strains. However, in some cases, there was also an association with the place of isolation of the strains. Our collection of the TBEV strains is not complete with respect to the different areas of the virus isolation. Most of recent strains come from the region of South Bohemia and therefore, we are not able to generalize the results to the whole Czech Republic. However, also the fine-scale TCS statistical parsimony analysis clearly showed the genetic evolutionary history of the virus in the past decades, as demonstrated on the results from the analysis of the most recent TBEV isolates included in this study and belonging to one phylogenetical clade (Fig. 2).

Interestingly, isolates 721, 760 and 828 from the years 1996, 1997 and 1999 were not grouped together with the most recent TBEV strains. These isolates were isolated in North Bohemia, in the district of Děčín, that represented latent focus region with no reported TBE human cases up to the year 1995. The grouping of these isolates more closely to the old strains could be explained by slower or restricted evolution of the virus in this region. The slower evolution can be caused by low level of the virus circulation in animal sphere or by solely vertical transmission of the virus in ticks due to a low density of the local tick population.

The degree of variation within European subtype of tick-borne encephalitis virus has been previously reported to be very low (Heinz et al., 1997; Ecker et al., 1999) that is in agreement with previously described antigenic homogeneity of TBEV (Heinz & Kunz, 1981, 1982; Guirakhoo *et al*., 1987). For example, isolates from Italy differed from the European prototype strain Neudoerfl only at eight nucleotide positions of the sequence of E gene. However, these substitutions were all synonymous and therefore there were no differences at the amino acid level (Hudson et al., 2001).

On the other hand, Casati et al. (2006) found high variability rate (55.5%) in the sequence of 5´UTR and C gene from TBEV strains isolated in an endemic area of Central Switzerland. Moreover, there was substantial heterogeneity in potential cyclization sequences and “start” codon in six strains from 44 sequenced (Casati et al., 2006).

In general, developmental changes in tick-borne flaviviruses are far less frequent than amongst those carried by mosquitoes (Ni et al.,1995; Nam et al., 1996; Lepiniec et al., 1994; Chu et al., 1989; Lewis et al., 1993; Lanciotti et al., 1997; Lanciotti et al., 1994). In fact, the tick-borne encephalitis virus is remarkably stable under natural conditions.

In our study, we observed minor genetic variations in the sequence of E gene within the time period of more than 50 years, exhibiting a maximum of only 1.3% at the amino acid level. The genetic variation observed between viruses was mainly due to synonymous point substitutions, the majority of which were transitions. Since 1948, there was accumulation of nucleotide substitutions in the sequence of E gene, but the number of amino acid substitutions did not show increasing tendency (Fig. 3), indicating that the observed amino acid changes were not able to resist the selection pressure and therefore were not preserved in the viral genome. Moreover, some of the identified amino acid substitutions were at positions previously described as affecting neuroinvasiveness or neurovirulence of various flaviviruses (Chambers

et al., 1998; Schlesinger et al., 1996; Chambers and Nickells, 2001; Hasegawa et al., 1992; Pletnev and Men, 1998). Beside this, we observed average d_N/d_S ratio strongly suggesting the positive selection on this gene in TBEV evolution. Protein E is the main component of the virus surface, probably mediating receptor binding and fusion activity after uptake by receptor mediated endocytosis. It seems that for its functional importance in the primary phase of the viral life cycle, this protein can undergo only minor sequence and structural changes. Even minor changes in the three-dimensional structure of the E protein may affect its ability to form heterodimers with prM or homodimers with E on the virion surface. More importantly, the ability of a receptor binding region to interact with its cognate receptor is likely to be severely disrupted by perturbations in the tertiary structure (Hurrelbrink and McMinn, 2003). Many molecular determinants of virulence of different flaviviruses have been identified in E protein previously. When analyzed in terms of their location on the three-dimensional structure of the protein, patterns begin to emerge that are suggestive of specific effects on structure and function (Hurrelbrink and McMinn, 2003). Superimposition of molecular determinants of virulence onto the TBE E protein model reveals five clusters of mutations: four located on the ectodomain of the protein (clusters A-D) and one located in the stem-anchor region (cluster E) (Hurrelbrink and McMinn, 2003). In this study, amino acid variation in the analyzed strains was identified at 25 positions. These changes fall into two clusters when superimposed in the 3D model of E protein. Amazingly, these clusters are corresponding to the previously defined clusters A and D. Mutations in cluster A are found predominantly on the lateral face of domain III (Fig. 4), a domain that has been implicated in receptor binding (cit). Region of cluster D is believed to be directly adjacent to the proposed position(s) for prM binding, as it is thought that prM forms heterodimer with protein E during virus assembly (Wengler and Wengler, 1989).

The region of 3' UTR is characterized by extensive length and sequential heterogeneity (Wallner et al., 1995). This part of viral genome can be divided into two parts: a proximal (localized behind the “stop” codon of the open reading frame) and a distal (“core”, the 3' terminus itself). The distal part of this region (approx. 340 nt) is highly conserved, whilst the proximal part is a noticeably variable segment with common deletions and insertions (Proutski et al., 1997; Gritsun et al., 1997). According to the study by Mandl et al. (1998), neither spontaneous nor engineered deletions in the hypervariable domain of TBEV 3’ UTR affected virus pathogenicity in cell culture or mice. One can therefore propose that this deletion did not contribute significantly to the pathogenetic properties of the virus. However, on the basis of analysis of higher number of sequences of 3’ UTR of strains from the same region, we found somewhat regularity in the character of the variable proximal segment. We identified three conservative and one semiconservative pattern of the deletions within the variable region with most prominent relatively short deletion affecting only poly(A) segment (Fig. 4). This pattern possibly represents the most frequent character of 3’UTR in wild strains and was observed also in other previously sequenced European as well as Siberian and Far-Eastern TBEV strains (Hayasaka et al., 2001). The other deletion patterns are less frequent, but also exhibit clear regularity. Deletions of defined length allow formation of distinct secondary structures of 3’ UTR that may play some role in properties of the virus under natural conditions. The 3’ UTR secondary structure plays an important role in viral RNA replication, behaving as the *cis*-acting signals for the initiation of transcription (You & Padmanabhan, 1999) and the specific binding site recognized by viral and cellular proteins (Chambers *et al*., 1990; Chen *et al*., 1997; Blackwell and Brinton, 1995, 1997; Ta & Vrati, 2000). Although the deletion inside the variable region does not affect virulence for laboratory mice, it can change the properties of the virus for the tick vector or natural hosts (Hayasaka et al., 2001). The identified regularity in the character of 3’ UTR strongly suggests its importance for the virus replication under natural conditions, or for the circulation of the virus in nature.

In conclusion, we performed comprehensive molecular epidemiological analysis of TBEV isolates from the time period of more than 50 years, which included strains belonging to the first isolated in Europe, strains from human samples, natural hosts as well as from two species of ticks. The study defined conclusively the phylogenetical and evolutional changes occurring within viral E gene that are discussed with respect to the epidemic activity of the virus and revealed conservative and semiconservative patterns of 3' UTR suggesting functional importance of both variable and core elements of 3' UTR for virus growth and/or circulation under natural ecological conditions.

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Authors contribution: DR planned the study, performed sequence analysis, interpreted results and wrote the manuscript, VD and JH isolated and collected most of the strains, TSG and EAG helped with the interpretation of the results and edited the manuscript on scientific and language basis, KK isolated some of the strains, JK and LG discussed and supervised the study.

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Tables and Figures

Table 1. Summary of TBEV strains used in this study.

Strain	Year of isolation	Passage history	Geographical origin	Source
Říha	**1948**	54 in mice	Czech Republic	human patient
Petráčová	**1953**	7 in mice	Czech Republic	human patient
Porodová	**1953**	7 in mice	Czech Republic	human patient
Hypr	**1953**	5 in mice	Brno, Czech Republic	human patient
414	**1954**	3 in mice	Poteplí, Czech Republic	*I. ricinus*
399	**1963**	3 in mice	Poteplí, Czech Republic	*I. ricinus*
398	**1963**	3 in mice	Poteplí, Czech Republic	*I. ricinus*
39	**1966**	3 in mice	Czech Republic	*I. ricinus*
40	**1966**	3 in mice	Poteplí, Czech Republic	*I. ricinus*
340	**1967**	1 in mice	Poteplí, Czech Republic	*I. ricinus*
465	**1967**	1 in mice	Poteplí, Czech Republic	Brain of dead squirrel *Sciurus vulgaris*
8641	**1977**	1 in mice	Rabyně, Czech Republic	Tick pool *I. ricinus* (100 nymphs)
9001	**1978**	1 in mice	Štěchovice, Czech Republic	Tick pool *I. ricinus* (177 nymphs)
9025	**1978**	1 in mice	Štěchovice, Czech Republic	Tick pool *I. ricinus* (11 males+11 females)
9045	**1978**	1 in mice	Čelina, Czech Republic	Tick pool *I. ricinus* (14 males+15 females)
62	**1986**	1 in mice	Kančinský-Brook, Czech Republic	Tick pool *I. ricinus* (7 males+ 5 females)
76	**1986**	1 in mice	Žďár, Czech Republic	Tick pool *I. ricinus* (22 males)
166	**1986**	1 in mice	České Budějovice, Czech Republic	Tick pool *I. hexagonus* (120 adults + 100 nymphs)
235	**1987**	1 in mice	Žďár, Czech Republic	Tick pool *I. ricinus* (4 females)
263	**1987**	2 in mice	Týn n.Vlt., Czech Republic	Tick pool *I. ricinus* (72 nyphs)
274	**1987**	1 in mice	České Budějovice, Czech Republic	Tick pool *I. ricinus* (6 males)
280	**1987**	1 in mice	Žďár, Czech Republic	Tick pool *I. ricinus* (2 nymphs)
282	**1987**	1 in mice	České Budějovice, Czech Republic	*I. ricinus* (female)
433	**1988**	1 in mice	Žďár, Czech Republic	Tick pool *I. ricinus* (7 males)
721	**1996**	1 in mice	Děčín, Czech Republic	Tick pool *I. ricinus* (25 males)
760	**1997**	1 in mice	Neznabohy, Czech Republic	Tick pool *I. ricinus* (14 females)
828	**1999**	1 in mice	Ústí nad Labem, Czech Republic	Tick pool *I. ricinus* (5 females)
200	**2000**	3 in PS cells, 1 in mice	Borovany, Czech Republic	Tick pool *I. ricinus* (20 nymphs)
206	**2000**	2 in PS cells, 1 in mice	Borovany, Czech Republic	Tick pool *I. ricinus* (10 males)
679	**2001**	3 in PS cells, 1 in mice	Borovany, Czech Republic	Tick pool *I. ricinus* (20 nymphs)
798	**2001**	3 in PS cells, 1 in mice	Římov, Czech Republic	Tick pool *I. ricinus* (20 nymphs)
1015	**2002**	2 in PS cells, 1 in mice	Borovany, Czech Republic	Tick pool *I. ricinus* (20 nymphs)
1025	**2002**	2 in PS cells, 1 in mice	Borovany, Czech Republic	Tick pool *I. ricinus* (20 nymphs)
1133	**2002**	2 in PS cells, 1 in mice	Hrádek, Czech Republic	Tick pool *I. ricinus* (20 nymphs)

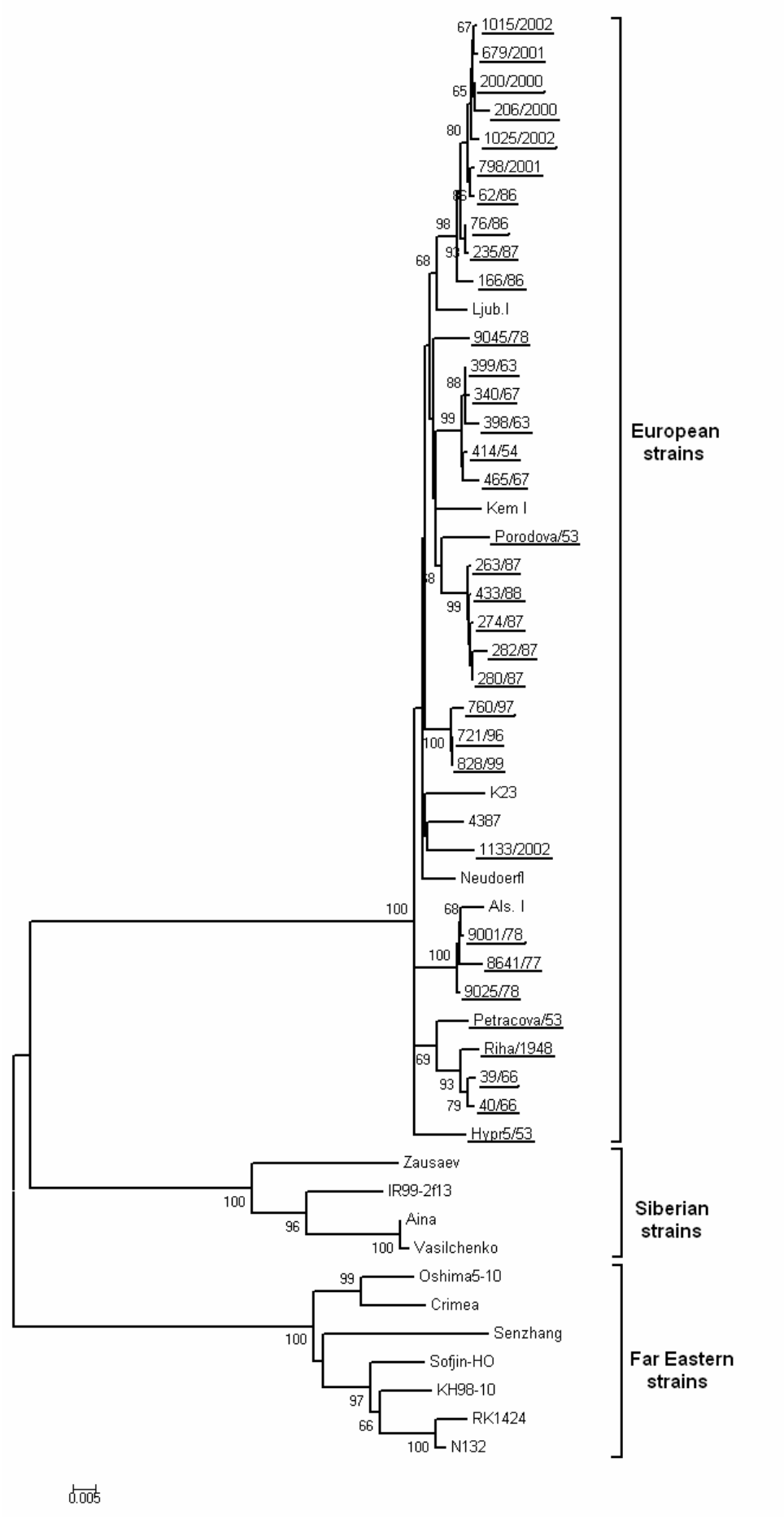
Table 2. List of previously sequenced TBEV strains from the region of Central Europe included in this study.

Strain	Year of isolation	Geographical origin	Source	Accession No.	Reference
Kem I	1952	Tatabanya, Hungary	*I. ricinus*	AF091011	Ecker et al. 1999
Neudoerfl	1971	Neudoerfl, Austria	*I. ricinus*	M27157	Mandl et al., 1988
Als. I	1975	Alsace, France	*I. ricinus*	AF091007	Ecker et al. 1999
K23	1975	Karlsruhe, Germany	*I. ricinus*	AF091010	Ecker et al. 1999
4387	1982	Gbelce, Slovakia	*Clethrionomys glareolus*	X76607	Labuda et al. 1994

Table 3. Amino acid differences identified in the studied strains after comparison to the prototypic strain Neudoerfl.

Position	Amino acid difference	Strain	
52	N→S	all strains with the exception: Porodova/53, Petracova/53, 39/66, 40/66, Riha/48, Hypr/54, 760/97, 721/96, 828/99, 1133/2002	
68	**D→P**	166/86	
81	T→I	8641/77, 9001/78, 9025/78, 1133/2002	
122	**E→G**	206/2000	
134	**A→V**	9045/78	
145	**P→S**	760/97	
167	I→V	all studied strains	
169	**S→T**	Riha/48, Petracova/53	
	S→L	280/87, 282/87	
195	**A→P**	1025/2002	
251	**K→R**	8641/77	
283	**L→Q**	465/67	
287	**H→P**	398/63	
291	E→K	282/87	
315	**K→R**	Porodova/53	
317	**A→V**	9045	FE-strains at this position I, S-strains T, for E non-conservative
331	T→S	39/66, 263/87, 274/87, 280/87, 282/87, 433/88	
333	**S→F**	Riha/48	
338	**C→G**	Porodova/53	
351	**D→A**	206/2000	
	D→Y	62/86	
352	**V→A**	398/63	
372	**I→V**	39/66, 40/66	
416	**I→M**	398/63	
433	I→V	Porodova/53	substitution typical for S- and FE-strains
437	V→L	Hypr	substitution typical for S- and FE-strains
462	V→L	679/2001	

non-conservative or unique substitututions are presented in bold


1015/2002
679/2001
200/2000
206/2000
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798/2001
62/86
76/86
235/87
166/86
Ljub.I
9045/78
399/63
340/67
398/63
414/54
465/67
Kem I
Porodova/53
263/87
433/88
274/87
282/87
280/87
760/97
721/96
828/99
K23
4387
1133/2002
Neudoerfl
Als. I
9001/78
8641/77
9025/78
Petracova/53
Riha/1948
39/66
40/66
Hypr5/53
Zausaev
IR99-2f13
Aina
Vasilchenko
Oshima5-10
Crimea
Senzhang
Sofjin-HO
KH98-10
RK1424
N132
European strains
Siberian strains
Far Eastern strains
0.005

Fig. 1. Neighbor-Joining tree showing the evolutionary relationships among the E gene sequence of the strains analyzed in this study (underlined) together with other isolates from Central Europe. Siberian and Far Eastern TBEV strains were used as outgroops. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Bootstrap values higher than 65% (1,000 replications) are shown. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

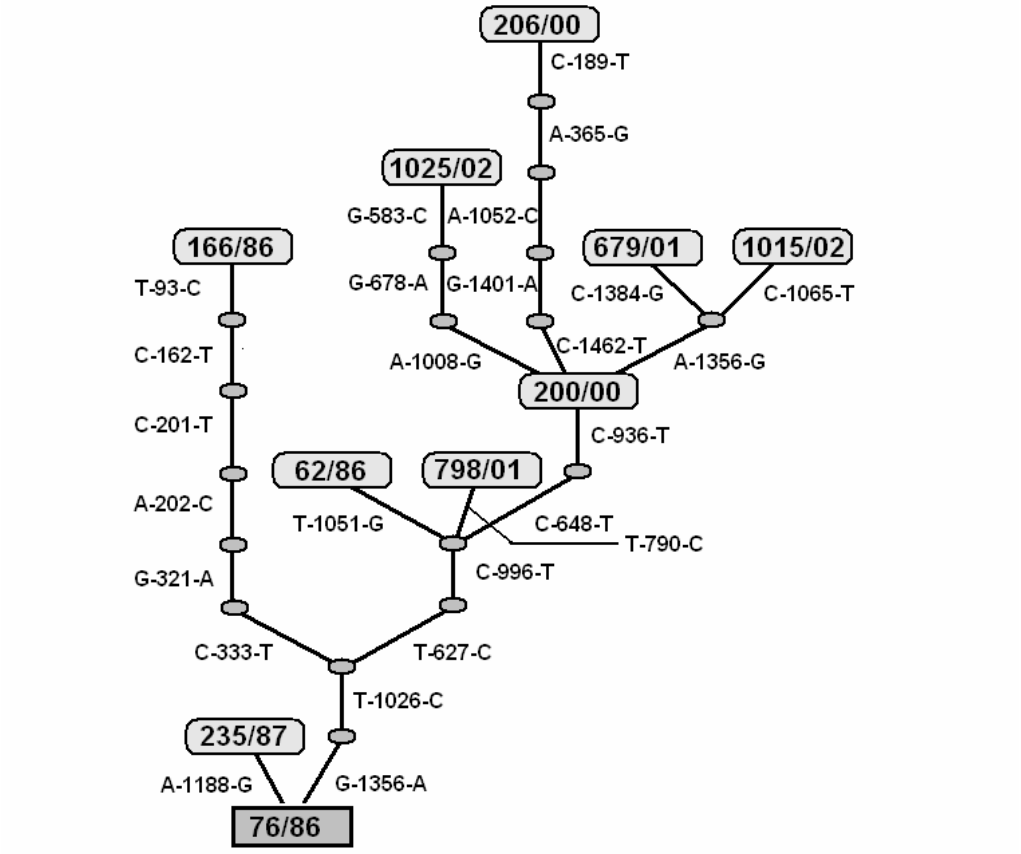

Fig. 2. Fine-scale statistical parsimony TCS analysis of 10 TBEV strains belonging to the cluster of most recent isolates from the time period of 1986-2002. Each connecting branch line represents a nucleotide substitution, with each dot representing a putative ancestor virus.

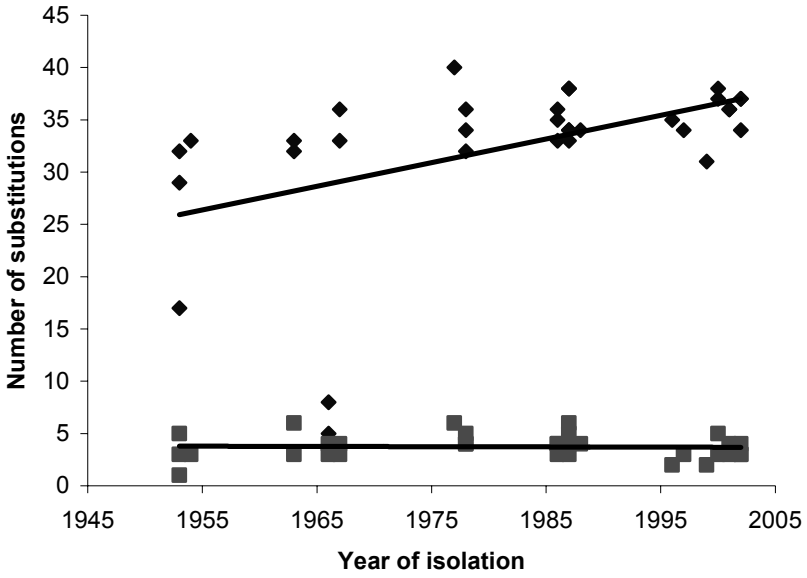

Fig. 3. Accumulation of substitutions with respect to time (nucleotide substitutions from the oldest isolate Riha/48). Shown is the accumulation of synonymous mutations (◆) increasing linearly with time; number of non-synonymous mutations (■) occurring in analyzed strains does not exhibit increasing tendency.

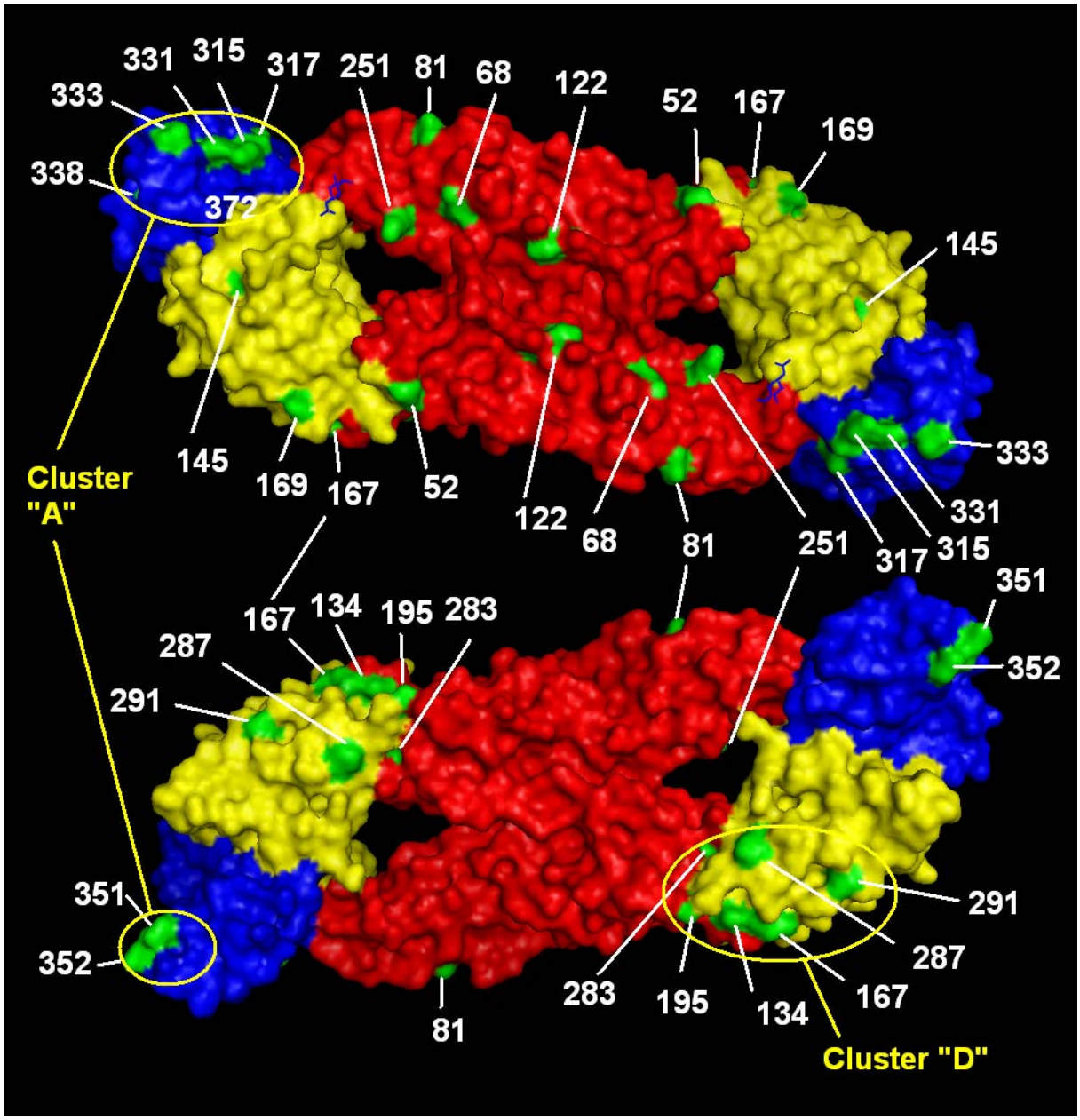

Fig. 4. Mapping of the positions of amino acid substitutions of the studied TBEV strains in the 3D structure of the E glycoprotein of TBEV strain Neudoerfl (Rey et al., 1995). The dimeric form of E protein molecule is presented in colours corresponding to domains I (yellow) and II (red) and III (blue). Domains were designed according to the model available in Protein Data Bank under the accession number 1SVB.pdb. Mutations are shown in green. Shown are both sides of the dimeric form of E protein.

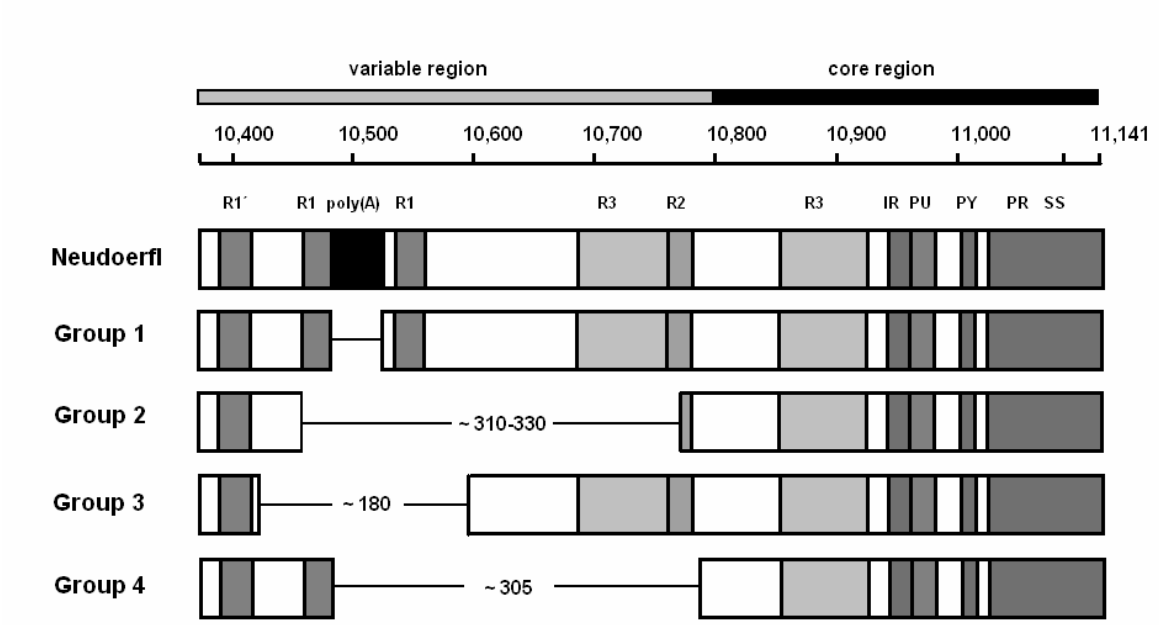

Fig. 5. Schematic drawing of the 3' UTR sequences of TBEV strains analyzed in this study and of the prototypic TBEV strain Neudoerfl. The 3' UTR is divided into a variable region and core segment, as described in the text. Numbering is according to the sequence of Neudoerfl strain. Designation of individual sequence elements corresponds to the literature (Wallner et al., 1995).

Chapter II.2

Rapid subtyping of tick-borne encephalitis virus isolates using multiplex RT-PCR.

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Rapid subtyping of tick-borne encephalitis virus-isolates using multiplex RT-PCR

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Summary
Tick-borne encephalitis virus, an emerging pathogen in several countries in Europe and Asia, has been divided into three subtypes (European, Siberian and Far Eastern). These subtypes are associated with the disease of different severity. For that reason, early distinguishing of the subtype in clinical sample or in ticks removed from the patient in areas of co-circulation of two or three subtypes is of high importance. For that reason, a simple method of multiplex RT-PCR for rapid and easy subtyping of tick-borne encephalitis virus isolates was developed. The method is based on the unique combination of oligonucleotide primers hybridizing with subtype-specific "signature" positions of the sequence encoding the viral envelope protein. The developed multiplex RT-PCR appears to be a useful method also in the studies focused on the molecular-epidemiology of tick-borne encephalitis virus.

Chapter II.3

Growth of tick-borne encephalitis virus (European subtype) in cell lines from vector and non-vector ticks.

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Growth of tick-borne encephalitis virus (European subtype) in cell lines from vector and non-vector ticks.

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Key words: tick-borne encephalitis virus, ticks, tick cell line, vectors

Abstract

We undertook a comparative study of the susceptibility of different tick cell lines to infection with the European subtype of tick-borne encephalitis virus (TBEV), prototype strain Neudoerfl. The growth of TBEV was investigated in lines derived from vector *Ixodes ricinus* L. ticks (IRE/CTVM18, 19, and 20), as well as non-vector ticks, namely *Ixodes scapularis* Say (IDE2), *Boophilus microplus* Canestrini (BME/CTVM2), *Hyalomma anatolicum anatolicum* Koch (HAE/CTVM9), *Rhipicephalus appendiculatus* Neumann (RA-257) and recently established and herein described lines from the argasid tick *Ornithodoros moubata* Murray (OME/CTVM21 and 22). All the tick cell lines tested were susceptible to infection by TBEV and the virus caused productive infection without any cytopathic effect. However, there was a clear difference between the TBEV growth in vector and non-vector cell lines, since *I. ricinus* cell lines produced 100-fold to 1,000-fold higher virus yield than the non-vector cell lines. The lowest virus production was observed in *O. moubata* and *R. appendiculatus* cell lines.

Chapter II.4

Mutations in the NS2B and NS3 genes affect mouse neuroinvasiveness of a Western European field strain of tick-borne encephalitis virus.

Daniel Růžek, Tamara S. Gritsun, Naomi L. Forrester, Ernest A. Gould, Jan Kopecký, Maryna Golovchenko, Nataliia Rudenko, Libor Grubhoffer.

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Mutations in the NS2B and NS3 genes affect mouse neuroinvasiveness of a western European field-strain of tick-borne encephalitis virus

Running title: Mouse neuroinvasiveness of tick-borne encephalitis virus

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Key words: tick-borne encephalitis virus; neuroinvasiveness; viral protease

Abstract
An attenuated strain (263) of tick-borne encephalitis virus, isolated from field ticks, was either serially subcultured, 5 times in mice, or at 40ºC in PS cells, producing 2 independent strains, 263-m5 and 263-TR with identical genomes; both strains exhibited increased plaque size, neuroinvasiveness and temperature-resistance. Sequencing revealed two unique amino acid substitutions, one mapping close to the catalytic site of the viral protease. These observations imply that virus adaptation from ticks to mammals occurs by selection of pre-existing virulent variants from the quasispecies population rather than by the emergence of new random mutations. The significance of these observations is discussed.

Chapter II.5

$CD8^+$ T-cells mediate immunopathology in tick-borne encephalitis.

Daniel Růžek, Jiří Salát, Tamara S. Gritsun, Ernest A. Gould, Iva Dyková, Jiří Jelínek, Jan Kopecký, Libor Grubhoffer

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CD8$^+$ T-cells mediate immunopathology in tick-borne encephalitis

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Key words: tick-borne encephalitis virus; immunopathology; CD8$^+$ T-cells; CD4$^+$ T-cells; SCID mice

Abstract

Epidemics of tick-borne encephalitis involving thousands of humans occur annually in the forested regions of Europe and Asia. Despite the importance of this disease, the underlying basis for the development of encephalitis remains undefined. Here, we prove the key role of CD8$^+$ T-cells in the immunopathology of tick-borne encephalitis, as demonstrated by prolonged survival of SCID or CD8$^{-/-}$ mice following infection in contrast with immunocompetent mice or mice with adoptively transferred CD8$^+$ T-cells. The results imply that tick-borne encephalitis is an immunopathological disease and that the inflammatory reaction significantly contributes to the fatal outcome of the infection.

Chapter II.6

Morphological changes in human neural cells following tick-borne encephalitis virus infection.

Daniel Růžek, Marie Vancová, Martina Tesařová, Arunee Ahantarig, Jan Kopecký, Libor Grubhoffer

will be submitted to *Journal of General Virology*

Morphological changes in human neural cells following tick-borne encephalitis virus infection.

Running Title: TBEV infection of human neural cells

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Abstract
Tick borne encephalitis (TBE) is one of the leading and most dangerous human viral neuroinfection in Europe and Asia. The clinical manifestations include asymptomatic infections, fevers and debilitating encephalitis that might progress into chronic disease or fatal infections. To further understand TBE pathology in host nervous system, three human neural cell lines, i.e. neuroblastoma, medulloblastoma and glioblastoma cells, were infected with TBE virus and the susceptibility and virus-mediated cytopathic effect including ultrastructural and apoptotic changes of the cells was examined.
All the neural cell lines tested were susceptible to TBEV infection. Interestingly, the neural cells produced about 100-10,000-fold higher virus titer than the conventional cell lines of extraneural origin, indicating the highly susceptible nature of the neural cells to TBEV infection.
The infection of medulloblastoma and glioblastoma cells was associated with number of major cytopathic changes including proliferation of membranous system and extensive rearrangement of cytoskeletal architecture. The areas of most intensive viral antigen signal were surrounded with high dense net of tubulins. The TBEV-infected cells were dying by both necrotic and apoptotic mechanisms. Observed ultrastructural apoptotic signs included condensation, margination and fragmentation of chromatin, vacuolation of the cytoplasm, dilatation of ER cisterns, and shrinkage of cells accompanied with high density of cytoplasm. On the other hand, infected neuroblastoma cells did not exhibit proliferation of membranous structures, virions were present in both endoplasmatic reticulum as well as in cytoplasm and the cells were dying preferentially by necrotic than apoptotic mechanism. Virological and neuropathological significance of these observations is discussed.

Chapter III.
Proposed future lines of research

The main aim of ongoing tick-borne encephalitis virus research is the development and application of practical and affordable disease control methods including cheaper vaccines and effective therapeutic tools, and improvements of the sensitivity and specificity of diagnostic/detection assays. A prerequisite for any of these aims is a detailed knowledge of the epidemiology of the disease in the target area, its (micro)evolution, identification of molecular determinants of biological properties of the causative agent, and last but not least, comprehensive understanding of the mechanisms of disease pathogenesis.
This thesis covers a broad range of topics related to TBEV research, including molecular epidemiology, laboratory detection, disease pathogenesis investigation and molecular biology.
However, the research is a never-ending story and each result opens new questions and challenges. Hence, also the results presented herein represent primarily a basis for number of ongoing or upcoming experiments and research projects that are summarized bellow.

As we demonstrated here, molecular epidemiological study has revealed that TBE virus is not subject to strong antigenic variation but is remarkably stable under natural ecological conditions. However, this study was primarily focused on the sequence variations of E gene. Upcoming full-length sequence analyses of the archival TBEV strains should bring more detailed information about evolution and selection pressure on the other TBEV structural and nonstructural genes and therefore allow more massive bioinformatic analysis of the results. This study will be realized in close collaboration with Robert Koch Institute in Berlin.
The developed method of multiplex RT-PCR allows extensive molecular-epidemiological studies focused on the distribution of TBEV in areas where co-circulation of the two or three subtypes occurs. The method, thanks to the simplicity, rapidity and inexpensiveness, allows investigating of large numbers of field-collected ticks and thus production of maps demonstrating distribution of separate TBEV subtypes, its overlays as well as association of different subtypes with certain tick species. Currently, some of these studies are in progress in Baltic States and Finland.
Our study focused on the interaction and growth of TBEV in various vector and non-vector tick cell lines brought some interesting results that need to be investigated in more detail in forthcoming projects. It was demonstrated previously that Langat virus, a flavivirus closely related to TBEV, has been successfully transmitted by *Ornithodoros* ticks under laboratory conditions (Turell and Durden, 1994; Turell et al., 2004). On the contrary, argasid ticks in general are not competent vectors of TBEV on the basis of major differences in TBEV interactions between ixodid and argasid species (Nuttall and Labuda, 1994). Here we demonstrated that the cell lines derived from argasid tick *Ornithodoros moubata* are sensitive to TBEV, although they produce low virus titer. However, by the day 10 post infection the cells had achieved virus titers nearly as high as in cells derived from the natural vector indicating adaptive microevolutionary changes occurring within the virus genome. Therefore, we would like to adapt TBEV to the *O. moubata* cells by serial subcultures in vitro. The subcultured TBEV variant will be investigated for its growth in the cell lines from argasid and ixodid ticks. Moreover, the attempt to infect *O. moubata* ticks and transmit the infection to laboratory mouse and/or to the next generation of *O. moubata* ticks with this variant will be carried out. Sequence analysis of E gene of the subcultured variant will be performed and compared with the parental strain. The possible sequence or antigenic differences will be analyzed with respect to the TBEV-vectorial competence of ixodid ticks.
Moreover, differences in the distribution of E protein antigen within the cells from various ticks may indicate differences in the maturation process of TBEV in these cells, and also this is

worth investigating. The maturation process will be investigated using transmission electron microscopy and cryo-electron tomography.

The pathogenesis of TBE remains still poorly understood. Here, we present a partial contribution to our understanding of the development of flavivirus encephalitis. Our contribution is based on the investigation of the role of host immune system in the development of the disease and on the study of the interaction of the virus with human neural cells. The results indicate that both topics are closely connected to each other; i.e. there is a balance between immunopathology and direct damage of neural cells by viral infection. In immunocompetent mice, we demonstrated detrimental effect of host $CD8^+$ T-cells accelerating the development and fatal outcome of the disease. On the other hand, in case of absence of immunity, the virus of its own was able to develop encephalitis and death of the experimental animals. Experiment in vitro with human neural cells revealed number of ultrastructural changes occurring after the infection, and, more importantly, revealed apoptosis as the main response to the TBEV infection.
At the cellular and molecular level, the factors determining TBEV development in neural cells are largely unknown. Further research is required to address questions such as: why the virus grows so rapidly in theses cells in comparison with extraneural cells, what are the receptors for TBEV, what changes in gene expression does the virus cause within these cells? What happens in the primary cultures of neural cells in comparison with neural cell lines? Does the virus infection cause apoptosis in human neurons and what is the balance between apoptosis and immunopathology? Why the virus infects glioblastoma cells, whereas glial cells from postmortem human tissue samples do not exhibit any reactivity with anti-TBEV monoclonal antibodies? Some of these questions may be answered thanks to the ongoing projects. Firstly, we are performing microarray analyses of changes in global gene expression within the neural cells infected with various high- and medium-virulent TBEV strains. Next to this, in the collaboration with the Faculty of Medicine of the Charles University in Hradec Králové, we will compare the results obtained from human neural cell lines with the results from the cultures of human neurons and glial cells derived from human dental pulp stem cells (Suchánek et al., 2007).

Taken together, the results presented in this thesis gave an insight into the various topics related with TBEV research, i.e. TBEV molecular epidemiology, microevolution, development of new detection methods, study of the interaction of the virus with tick cells, and the investigation of selected aspects TBE pathogenesis in vivo and in vitro. Although this research revealed some new issues, many questions remain unanswered and await further investigation.

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Chapter IV.
Shrnutí

Předkládaná disertační práce je zaměřena na studium molekulární epidemiologie a patogeneze klíšťové encefalitidy. Práci tvoří několik studií zaměřených na vybrané aspekty dané problematiky. Práce zahrnuje studie týkající se molekulární evoluce viru klíšťové encefalitidy (KE) ve střední Evropě od prvního objevení viru v roce 1948 až po současnost. Dále se zabývá mikroevolucí viru v buněčné kultuře a v laboratorních zvířatech, studiem interakce viru KE s buňkami odvozenými od různých vektor-kompetentních a nekompetentních klíšťat a v neposlední řadě studiemi zaměřenými na patogenezi klíšťové encefalitidy se zvláštním zřetelem na úlohu hostitelského imunitního systému při rozvoji encefalitidy a na interakci viru s lidskými neurálními buňkami.

(i) Studie zaměřená na molekulární evoluci viru KE ve střední Evropě byla založena na sekvenční analýze genu pro virový obalový protein a 3´ nekódujících úseků u souboru archivních kmenů viru KE pocházejících z rozmanitých zdrojů (lidské vzorky, klíšťata, rezervoárová zvířata). Analýza odhalila jen drobné genetické variace v genomu viru, ke kterým docházelo během posledních 50 let. Aminokyselinové záměny pozorované u různých kmenů však nebyly distribuovány pravidelně v rámci celé sekvence, ale spíše se shlukovaly v oblastech, které již dříve byly popsány coby oblasti zodpovídající za virulenci flavivirů, včetně oblasti, která je považována za potenciální receptor-vázající doménu. Dále byl v této studii popsán semikonzervativní charakter 3´ nekódující oblasti, což v kontrastu s dříve publikovanými pracemi naznačuje funkční význam celého 3´ nekódujícího úseku pro úspěšnou cirkulaci viru v přírodě (**Kapitola II.1**).

(ii) Pro účely molekulárně-epidemiologického výzkumu, případně i pro laboratorní diagnostiku jsme vyvinuli metodu multiplexové polymerázové řetězové reakce, která je schopna velmi rychle a snadno přiřadit daný analyzovaný izolát k jednomu ze tří subtypů viru KE. Jednotlivé subtypy se navzájem liší svými biologickými vlastnostmi, zejména s ohledem na virulenci. Proto je takováto subtypová charakterizace izolátů, zejména v oblastech vyznačujících se překryvem jednotlivých subtypů, velmi potřebná (**Kapitola II.2**).

(iii) Následně jsme provedli sledování interakce viru KE a různých klíštěcích buněk odvozených od vektor-kompetentních, ale i nekompetentních klíšťat. Pozorovali jsme, že virus KE se nejlépe množí v buňkách odvozených od klíštěte obecného *Ixodes ricinus*, přičemž nejméně v buňkách klíštěte *Rhipicephalus appendiculatus* či „měkkého“ klíštěte *Ornithodoros moubata.* Tato pozorování zřejmě souvisí s ideální adaptací viru ke svému vektoru, coby výsledku mnohatisícileté koevoluce. Nicméně poměrně rychle se podařilo virus adaptovat k buňkám nevektorových klíšťat, což může naznačovat, že v případě, kdy by virus byl nucen obsadit jinou ekologickou niku, by se poměrně rychle dokázal zřejmě adaptovat na jiná klíšťata a využívat je jako své vektory (**Kapitola II.3**).

(iv) Jeden kmen viru KE izolovaný z klíštěte *Ixodes ricinus* v jižních Čechách, který se vyznačoval neobvyklými biologickými vlastnostmi (atenuace, termosenzitivita), jsme podrobili důkladnějšímu zkoumání. Pozorovali jsme, že tento kmen není tvořen homogenní virovou populací, ale že v rámci tohoto kmenu je minoritně přítomna poměrně vysoce virulentní varianta. Tuto variantu jsme následně selektovali dvěma nezávislými postupy a podrobili ji kompletní genomové sekvenční analýze. Pozorovali jsme, že ona virulentní varianta se od parentální atenuované liší jen ve dvou aminokyselinových substitucích, které zřejmě alterují funkci virové proteázy. Tyto aminokyselinové substituce jsou tudíž pokládány za nově identifikované molekulární determinanty virulence viru KE (**Kapitola II.4**).

(v) Naše studium patogeneze klíšťové encefalitidy bylo realizováno ve dvou samostatných experimentech, které však spolu úzce souvisely. Předně jsme se zabývali úlohou hostitelského imunitního systému v patogenezi KE. Obecně se předpokládá, že zánětlivá reakce v mozku

sehrává úlohu v protekci před letální infekcí způsobenou neurovirulentními kmeny. Nicméně, my na základě série experimentů s různými kmeny myší, knockouty v genech pro T- a B-lymfocyty, či specifickou $CD8^+$ T-lymfocytů subpopulaci, a po provedení adoptivních přenosů subpopulací $CD4^+$ a $CD8^+$ T-lymfocytů, jsme pozorovali, že KE je primárně zřejmě imunopatologické onemocnění, tj. že náš vlastní imunitní systém (zejména tedy subpopulace $CD8^+$ T-lymfocytů) napomáhá fatálnímu výsledku infekce virem KE (**Kapitola II.5)**.
Druhá studie byla zaměřena na sledování interakce viru KE s lidskými neurálními buňkami. Ukázalo se, že kromě imunopatologie sehrává významnou roli v progresi KE i samotné poškození nervových buněk virovou infekcí, zejména virem indukovaná apoptóza (**Kapitola II.6**).

Summary

The dissertation thesis is focused on the investigation of molecular epidemiology and pathogenesis of tick-borne encephalitis (TBE). The thesis includes studies on molecular evolution of tick-borne encephalitis virus (TBEV) in Central Europe since the discovery of the virus up to the present (1948-2002), investigation of the microevolution of the virus in laboratory animals and cell cultures, interaction of the virus with cell lines derived from various vector and non-vector ticks, and studies on the pathogenesis of the disease with special regard to the role of host immune system in the development of encephalitis and to the interaction of the virus with neural cells.
(i) The study focused on the molecular epidemiology of TBEV in Central Europe was based on the complete sequence analysis of envelope (E) protein gene and 3' non-coding region. The study revealed minor genetic variations of the virus within the time period of more than 50 years, exhibiting a maximum of only 1.3% at the amino acid level. However, amino acid substitutions identified in the archival strains were not distributed randomly within the sequence of the protein E, but most of them were clustering into two areas of mutations affecting virulence of flaviviruses, including the potential receptor-binding domain. In contrast to previously published studies, we observed s semi-conservative character of the 3' untranslated region. The uniformity of deletions within 3'UTR suggests its importance for the survival of the virus under natural ecological conditions (**Chapter II.1**).
(ii) We developed a method of multiplex RT-PCR for rapid and easy subtyping of tick-borne encephalitis virus isolates. The three TBEV subtypes are associated with different severities of the disease. For that reason, early determination of the subtype in a clinical sample or in ticks removed from a patient in areas of co-circulation of two or three subtypes is of high importance. The developed method also appears to be a useful method in studies focused on the molecular-epidemiology of tick-borne encephalitis virus (**Chapter II.2**).
(iii) We also undertook a comparative study of the susceptibility of different tick cell lines to infection with the European subtype of tick-borne encephalitis virus. The growth of TBEV was investigated in lines derived from vector as well as non-vector ticks. All the tick cell lines tested were susceptible to infection by TBEV and the virus caused productive infection without any cytopathic effect. However, there was a clear difference between the TBEV growth in vector and non-vector cell lines, since *I. ricinus* cell lines produced 100-1000-fold higher virus yield than the non-vector cell lines. The lowest virus production was observed in *Ornithodoros moubata* and *Rhipicephalus appendiculatus* cell lines (**Chapter II.3**).
(iv) One TBEV strain isolated from *Ixodes ricinus* ticks collected in South Bohemia with peculiar biological properties (attenuation, temperature-sensitivity) was characterized in more detail. We observed that this strain is not formed by homogenous viral population, but that a highly virulent variant was present in the "quasispecies" of the strain. Subsequently, this variant was selected using two independent approaches and complete genomic sequence

analysis was performed. We revealed that the attenuated and highly virulent variants differ from each other only in two amino acid substitutions, altering the function of viral protease. Therefore, these substitutions are considered as newly identified molecular determinants of virulence of TBEV (**Chapter II.4**).

(v) Our investigation of the pathogenesis of TBE was realized in two separate studies that were, however, closely connected to each other. Firstly, we investigated the role of host immune system in the pathogenesis of TBE. It is generally believed that the inflammatory reaction in CNS is a requirement in the protection from lethal infection caused by neurovirulent strains. However, on the basis of experiments with different breeds of laboratory mouse, knockouts in genes coding for B- or T-cells or specific subpopulation of $CD8^+$ T-cells, and after the adoptive transfer of specific immune cell-populations ($CD4^+$ a $CD8^+$ T-cells), we revealed that TBE is primarily an immunopathological disease, i.e. that immune system plays a detrimental role, that is mediated especially by $CD8^+$ T-cells (**Chapter II.5**).

Secondly, we investigated the interaction of TBEV with human neural cells. This study revealed that direct damage of neural cells caused by virus infection plays also a crucial role in the development of the disease in addition to immunopathology. The infection was associated with number of major cytopathic changes including proliferation of membranous system and extensive rearrangement of cytoskeletal architecture and the TBEV-infected cells were dying by both necrotic and apoptotic mechanisms (**Chapter II.6**).

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List of abbreviations

CNS	central nervous system
CPE	cytopathic effect
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
E-	European
ER	endoplasmic reticulum
FE-	Far Eastern
GA	Golgi apparatus/complex
i.n.	intranasal
JEV	Japanese encephalitis virus
MBFV	mosquito-borne flaviviruses
m.o.i.	multiplicity of infection
MST	mean survival time
MVEV	Murray Valley encephalitis virus
PBS	phosphate-buffered saline
pfu	plaqu-forming unit
p.i.	post-infection
RER	rough endoplasmatic reticulum
S-/SIB	Siberian
s.c.	subcutaneous
SMS	smooth membrane vesicles
TBE	tick-borne encephalitis
TBEV	tick-borne encephalitis virus
TBFV	tick-borne flaviviruses
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling
UTR	untranslated/noncoding region
WE-	West European
WNV	West Nile virus

Curriculum vitae

Date and place of birth: April 30, 1981, Jindřichův Hradec, Czech Republic
Nationality: Czech
Marital Status: Single

Education:

2005-present **PhD. studies in Molecular and Cellular Biology and Genetics** at the Faculty of Science, University of South Bohemia and Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic
Dissertation: "Molecular epidemiology and pathogenesis of tick-borne encephalitis".
Supervisors: Prof. Libor Grubhoffer, Assoc. Prof. Jan Kopecký, Institute of Parasitology, Academy of Sciences of the Czech Republic

2007 ***Doctorate viva voce*** **examination**, Faculty of Biological Sciences, University of South Bohemia
Degree: ***Doctor rerum naturalium*** **(RNDr.)**
Thesis: "Rapid subtyping of tick-borne encephalitis virus using multiplex RT-PCR"
Supervisors: Prof. Libor Grubhoffer, Assoc. Prof. Jan Kopecký, Institute of Parasitology, Academy of Sciences of the Czech Republic

2003-2005 **Studies in Clinical Biology**, Faculty of Biological Sciences, University of South Bohemia
Degree: **Master of Science (*cum laude*)**
Thesis: "Genetic variability in tick-borne encephalitis virus".
Supervisors: Prof. Libor Grubhoffer, Assoc. Prof. Jan Kopecký, Institute of Parasitology, Academy of Sciences of the Czech Republic

2000-2003 **Studies in Biology-Biomedical Laboratory Techniques** at the Faculty of Biological Sciences, University of South Bohemia
Degree: **Bachelor of Science**
Thesis: "Use of real-time PCR in the quantitative detection of herpetic viruses".
Supervisors: Eva Zampachova, M.D., Natalja Piskunova, PhD., Department of Virology, Regional Hospital in České Budějovice

1994-2000 Gymnázium in Český Krumlov (6-years High School, General Education)

Clinical Fellowships

2002 Department of Virology, Regional Hospital in Ceske Budejovice
2003 Department of Bacteriology, Regional Hospital in Ceske Budejovice
Department of Virology, Regional Hospital in Ceske Budejovice
2004 Department of Clinical Immunology, Regional Hospital in Ceske Budejovice

Research Visits/Stays Abroad

2006 Royal (Dick) School of Veterinary Studies, Centre for Tropical Veterinary Medicine, University of Edinburgh, United Kingdom
2008 Laboratoire de Physiopathologie de la Barriere Hemato-Encephalique, Faculté des Sciences Jean Perrin, Université d'Artois, Lens, France.

Employment

Assistant, Faculty of Biological Sciences, University of South Bohemia (2004-2006)

Research Assistant, Institute of Parasitology Academy of Sciences of the Czech Republic (2005-present)

Lecturer (Medical Virology), Department of Medical Biology, Faculty of Science, University of South Bohemia (2006-present)

Certification

Certificate of competency according to §17 of the Act No. 246/1992 coll. on Protection Animals against Cruelty in present statues at large. University of Veterinary and Pharmaceutical Studies, Faculty of Veterinary Medicine, Brno (2005).

Certificate of Completion: International Course "Summer School on Molecular Vector Biology" (Ceske Budejovice, 2005).

Honors

1st Prize, Student Scientific Work 2000, South Bohemia Region, Czech Republic. Thesis: Molecular Biology of Oncogenic Viruses.

Special Award, Czech National Round of the Student Scientific Work 2000, Rožnov pod Radhoštem, Czech Republic.

1st Prize, Student Scientific Work 2006, Student Grant Agency of the Faculty of Biological Sciences, University of South Bohemia.

Member of the Czechoslovak Society for Microbiology (since 2008)

List of publications

Publications in journals with impact factor:

Růžek, D., Bell-Sakyi, L., Kopecký, J., Grubhoffer, L. 2008. Growth of tick-borne encephalitis virus (European subtype) in cell lines from vector and non-vector ticks. *Virus Research* 137, 142-146.

Rudenko, N., Golovchenko, M., Mokráček, A., Piskunova, N., Růžek, D., Mallátová, N., Grubhoffer, L. 2008. Detection of *Borrelia bissettii* in cardiac valve tissue of a patient with endocarditis and aortic valve stenosis (Czech Republic). *Journal of Clinical Microbiology*, in press, published on-line doi:10.1128/JCM.01032-08.

Růžek, D., Gritsun, T.S., Forrester, N.L., Gould, E.A., Kopecký, J., Golovchenko, M., Rudenko, N., Grubhoffer, L. 2008. Mutations in the NS2B and NS3 genes affect mouse neuroinvasiveness of a Western European field strain of tick-borne encephalitis virus. *Virology* 10, 249-255.

Růžek, D., Piskunova, N., Žampachová, E. 2007. High variability in viral load in cerebrospinal fluid from patients with herpes simplex and varicella-zoster infections of the central nervous system. *Clinical Microbiology and Infection* 13, 1217-1219.

Růžek, D., Šťastná, H., Kopecký, J., Golovljova, I., Grubhoffer, L. 2007. Rapid subtyping of tick-borne encephalitis virus isolates using multiplex RT-PCR. *Journal of Virological Methods* 144, 133-137.

Růžek, D., Štěrba, J., Kopecký, J., Grubhoffer, L. 2006. The supposedly attenuated hy-HK variant of highly virulent Hypr strain of tick-borne encephalitis virus is obviously a strain of Langat virus. *Acta Virologica* 50, 277-278.

Submitted papers:

Růžek, D., Salát, J., Gritsun, T.S., Gould, E.A., Dyková, I., Jelínek, J., Kopecký, J., Grubhoffer, L. 2008. $CD8^+$ T-cells mediate immunopathology in tick-borne encephalitis. *Virology*, submitted 27. May 2008, in revision.

Rudenko, N., Golovchenko, M., Růžek, D., Piskunova, N., Mallátová, N., Grubhoffer, L. 2008. Molecular detection of *Borrelia bissettii* DNA in serum samples from the patients with symptomatic borreliosis or chronic borrelial infection in the Czech Republic. *FEMS Microbiology Letters*, submitted 1. August 2008.

Abstracts in journals with impact factor:

Růžek, D., Šťastná, H., Kopecký, J., Grubhoffer, L. 2006. Multiplex RT-PCR for detection and subtyping of tick-borne encephalitis virus. *Journal of Clinical Virology* 36, S32.

Růžek, D., Kopecký, J., Štěrba, J., Golovchenko, M., Rudenko, N., Grubhoffer, L. 2006. Non-virulent strains of TBE virus circulating in the Czech Republic. *Journal of Clinical Virology* 36, S41.

Rudenko, N., Golovchenko, M., Piskunova, N., Růžek, D., Grubhoffer, L. 2008. Detection of Borrelia bissettii in the South Bohemia region of the Czech Republic: cases of single and multiple infections in humans. *Clinical Microbiology and Infection* 14, S109-S110.

List of conferences

Invited lectures:
Růžek, D. et al. Molecular epidemiology and pathogenesis of tick-borne encephalitis. Spiez Laboratory, Federal Department of Defence, Civil Protection and Sports DDPS, Federal Office for Civil Protection FOCP, Spiez, Switzerland. May 8, 2008.

Růžek, D., et al. Pathogenesis of tick-borne encephalitis. University d'Artois, Lens, France. January 18, 2008.

Růžek, D., et al. Molecular epidemiology and pathogenesis of tick-borne encephalitis. Robert-Koch Institute, Berlin, Germany. March 12, 2007.

Růžek, D., et al. Identification of two potential molecular determinants of an attenuated temperature-sensitive phenotype of tick-borne encephalitis virus. RNA Club. České Budějovice, Czech Republic. October 3, 2005.

invited lectures given in Czech:
Růžek, D. Epidemiology of tick-borne encephalitis. Epidemiology of selected parasital diseases. Prague, Czech Republic. April 1, 2008.

Posters at international conferences
Růžek, D. et al., Molecular neuropathogenesis of tick-borne encephalitis. XIV. International Congress of Virology, Istanbul, Turkey. August 10-15, 2008.

Šťastná, H., Růžek, D. et al. Molecular interaction of tick-borne encephalitis virus with human neural cells. XIV. International Congress of Virology, Istanbul, Turkey. August 10-15, 2008.

Bell-Sakyi, L., Růžek, D., Gould, E.A. Cell lines from the soft tick *Ornithodoros moubata*. VI International Conference on Ticks and Tick-borne Pathogens, Buenos Aires, Argentina. September 21-26, 2008.

Šťastná, H., Růžek, D. et al. The influence of tick saliva on the replication of tick-borne encephalitis virus. Third European Congress of Virology, Nürnberg, Germany. September 1-5, 2007.

Růžek, D. et al. Interaction of tick-borne encephalitis virus with human cells of neural origin. Third European Congress of Virology, Nürnberg, Germany. September 1-5, 2007.

Růžek , D et al., Immunopathological features of tick-borne encephalitis. Third European Congress of Virology, Nürnberg, Germany. September 1-5, 2007.

Růžek, D. et al. Molecular evolution of tick-borne encephalitis virus in the Central Europe over 50 years. Third European Congress of Virology, Nürnberg, Germany. September 1-5, 2007.

Růžek, D. et al. Multiplex RT-PCR for detection and subtyping of tick-borne encephalitis virus. Joint Meeting of the European Society for Clinical Virology and the Clinical Virology Group of the Society for General Microbiology, Birmingham, United Kingdom. September 3-6, 2006. [participation supported by Travel Grant from the European Society for Clinical Virology].

Růžek, D. et al. Non-virulent strains of TBE virus circulating in the Czech Republic. Joint Meeting of the European Society for Clinical Virology and the Clinical Virology Group of the Society for General Microbiology, Birmingham, United Kingdom. September 3-6, 2006. [participation supported by Travel Grant from the European Society for Clinical Virology].

Růžek, D. et al. Characterization of a virulent variant selected from a quasispecies of an attenuated strain of tick-borne encephalitis virus. 5th International Conference on Ticks and Tick-borne Pathonens. Neuchatel, Switzerland. August 29- September 2, 2005.

Růžek, D. et al. Identification of two potential molecular determinants of an attenuated temperature-sensitive phenotype of tick-borne encephalitis virus. 10th International Conference on Lyme borreliosis and other Tick-borne pathogens, Vienna, Austria. September 11-15, 2005.