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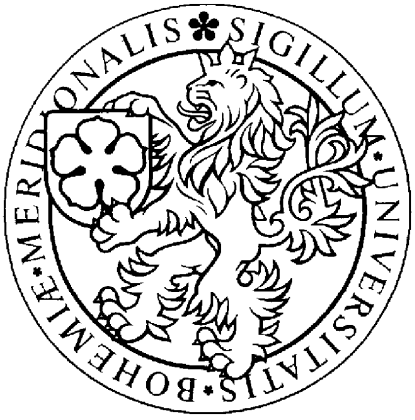

Rapid subtyping of tick-borne encephalitis virus-isolates using multiplex RT-PCR

RNDr. Thesis

Mgr. Daniel Růžek

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Annotation:
Early determination of the subtype of tick-borne encephalitis virus in a clinical sample or in ticks in areas of co-circulation of two or three subtypes is of high importance. The development of a simple method of multiplex RT-PCR for rapid and easy subtyping of tick-borne encephalitis virus isolates is reported to fill this requirement. 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 also appears to be a useful method in studies focused on the molecular-epidemiology of tick-borne encephalitis virus.

Anotace:
Včasné rozlišení subtypu viru klíšťové encefalitidy v klinických vzorcích či klíšťatech je velmi významné zejména v oblastech vyznačujících se současným výskytem dvou nebo všech třech subtypů. V této práci je prezentována nová jednoduchá metoda založená na multiplex RT-PCR pro rychlou subtypizaci izolátů viru klíšťové encefalitidy. Metoda je založena na unikátní kombinaci oligonukleotidových primerů hybridizujících se subtypově-specifickými pozicemi v sekvenci kódující virový obalový protein E. Vyvinuté metody bude nejspíše využito též v molekulárně-epidemiologickém výzkumu viru klíšťové encefalitidy.

The project was supported by the grants Z60220518, MSM 6007665801 of the Ministry of Education, Youth and Sports of the Czech Republic, the grant 524/06/1479 from the Grant Agency of the Czech Republic, Research Centre of the Ministry of Education and Youth and Sports of the Czech Republic No. LC 06009, FRVS 230/2007, 44/2006/P-BF of the Grant Agency of the University of South Bohemia and grant 5963 of the Estonian Science Foundation.

I hereby declare that my contribution to the published study that constitutes this RNDr. Thesis was my own work and that I used only cited literar sources.

……..
Mgr. Daniel Růžek

České Budějovice Apr 27, 2007

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Confirmation

I hereby certify the principal participation of Mgr. Daniel Růžek on the publication: Růžek et al. 2007. Rapid subtyping of tick-borne encephalitis virus-isolates using multiplex RT-PCR, accepted for publication in *Journal of Virological Methods*.

Irina Golovljova, MD. PhD.

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Journal of Virological Methods

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 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 development of a simple method of multiplex RT-PCR for rapid and easy subtyping of tick-borne encephalitis virus isolates is reported to fill this requirement. 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 also appears to be a useful method in studies focused on the molecular-epidemiology of tick-borne encephalitis virus.

Key-words: tick-borne encephalitis virus, subtyping, ticks, multiplex RT-PCR

1. Introduction

Since its introduction, multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics, including the detection of RNA. In the field of infectious diseases, the technique has been shown to be a valuable method for identification of various pathogens: viruses, bacteria, fungi and parasites (Elnifro et al., 2000).

One of the most dangerous human viral neuroinfections in Europe and Asia is tick-borne encephalitis (TBE), a disease caused by tick-borne encephalitis virus (TBEV). Thousands of people are infected with TBEV and many deaths are reported annually (Gritsun et al., 2003a).

TBEV is a member of the genus *Flavivirus* within the family *Flaviviridae*. TBEV is an enveloped virus containing a single positive stranded RNA genome approximately 11 kb in length, consisting of a single open reading frame (ORF), flanked by 5' and 3' non-coding regions. The ORF encodes a single polyprotein that is cleaved by viral and cellular proteases into 10 proteins: three structural proteins (C, prM, E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Chambers et al., 1990).

Based on the sequence encoding the viral envelope (E) protein, TBEV has been subdivided into 3 subtypes: European, Far Eastern and Siberian (Ecker et al., 1999). Interestingly, these three subtypes are associated with different severities of the disease. Encephalitis caused by members of the European subtype (previously Central European Encephalitis) is usually mild, with case fatality rate reaching 1-5 % (Grešíková and Kaluzová, 1997). On the other hand, Far Eastern strains produce very severe encephalitis (previously Russian Spring-Summer Encephalitis), with a fatal outcome in 20 – 60 % cases (Gritsun et al., 2003a; Dumpis et al., 1999). TBEV strains from the Siberian region cause a less severe disease compared to that of the Far Eastern subtype, with a tendency to develop chronic or extremely prolonged infections (Gritsun et al., 2003b), but the case fatality rates rarely exceed 6 – 8 % (Gritsun et al., 2003a).

Co-circulation of members of all three TBEV subtypes was reported in the Baltic States (Lundkvist et al., 2001; Golovljova et al., 2004) and members of the European and Siberian subtype also co-circulate in adjacent countries such as Finland (Jääskeläinen et al., 2006). Theoretically, the TBEV subtypes co-circulate in broad areas of parallel occurrence of the two major tick vectors of TBEV: *Ixodes ricinus*, the main vector of members of the European subtype and *I. persulcatus*, the principal vector of the Siberian and Far Eastern strains.

Rapid subtyping of TBEV in clinical specimens or in ticks removed from the patient is important for assessing the prognosis of the disease in these areas. Moreover, molecular-epidemiological research on the circulation of the TBEV subtypes in different areas of Eurasia is unconceivable without a simple and reliable method of subtyping TBEV isolates.

2. Materials and Methods

2.1 Primer design

A set of six oligonucleotide primers was selected on the basis of comparative sequence analysis of all the currently sequenced TBEV isolates. The primers hybridize specifically with subtype-specific "signature" positions of the sequence encoding the viral envelope (E) protein (Fig. 1). The universal "antisense" primer (5´-CTC ATG TTC AGG CCC AAC CA-3´) for reverse transcription was selected on the basis of a region of high sequence homology in all members of all TBEV subtypes. The sequence and positions of the primers are given in Table 1.

2.2 Viruses

Members of all three TBEV subtypes were used in this study. Strain Hypr (GenBank accession number **U39292**; Pospíšil et al., 1954; Wallner et al., 1996), a member of the European subtype; strain Sofjin, a member of the Far Eastern subtype (**X07755**; Zilber, 1939), and a member of the Siberian subtype Est54 (**DQ393773**; Golovljova et al., 2004) were used for the optimization of the protocol, infection of mice and ticks and preparation of clinical samples. TBEV strains 263 (**U27491**) and 280 (**EF113085**) isolated from the ticks *Ixodes ricinus* (Růžek et al., 2006) and the strain 166 (**EF113079**) from *Ixodes hexagonus* collected in the south of the Czech Republic were analyzed using the multiplex RT-PCR procedure. Yellow fever virus 17D (Rice et al., 1985), human herpes simplex virus type 1 and 2 and human enterovirus 71 (Schmidt et al., 1974) were assayed by multiplex RT-PCR, in order to exclude the possibility that the assay nonspecifically detects another flavivirus or other viruses important for differential diagnosis in CNS diseases.

2.3 Preparation of analyzed samples

Samples from different sources were analyzed by the newly developed multiplex RT-PCR. Adult laboratory-bred ticks *I. ricinus* were infected by feeding on TBEV-positive adult laboratory mice. Ticks were allowed 12 hours to feed and then the mice were

inoculated subcutaneously with 1.000 plaque forming units (PFU) of TBEV, strain Hypr. After full engorgement of the ticks, they were kept for 14 days at normal laboratory temperature and higher relative humidity to digest the blood. Afterwards, ticks in pools of 3-4 individuals were homogenized with a mortar and pestle in 0.5 ml of L-15 cultivation medium (Sigma-Aldrich) supplemented with 3% of fetal calf serum, and RNA was isolated from the homogenate.

Suspensions of ticks *I. persulcatus* (collected in nature; Novosibirsk Region, Russia) were prepared and varying titers of TBE virus (Hypr, Sofjin or Est54) were added to the tick homogenates.

Organs (brain, spleen) of laboratory mice subcutaneously infected with 100 PFU of TBEV (strains Hypr, Sofjin or Est54) and exhibiting marked neurological symptoms were prepared as 20% ($w.v^{-1}$) suspensions in L-15 medium with 3% fetal calf serum.

Model clinical samples were prepared by addition of different amounts ($1x10^6$, $1x10^4$, $1x10^2$) of TBE virus (Hypr, Sofjin, Est54) to the serum from a healthy blood donor.

2.4 Isolation of RNA and reverse transcription

Viral RNA was isolated using the QIAamp Viral RNA Kit (Qiagen), according to the recommendations of the manufacturer. The RNA was eluted with 60 μL of the buffer available in the kit. 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 amplification, using 1 μL of the "antisense" primer (0.5 μM).

2.5 PCR conditions

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), 1 μL of template. The cycling conditions were as follows: 5 min of denaturation at 95 °C, followed by 30 cycles with 95 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min 30 s. The results were visualized by performing 1.7 % agarose gel electrophoresis in Tris-acetate-EDTA buffer.

3. Results

A set of oligonucleotide primer pairs was selected to distinguish TBEV subtypes by production of three subtype-specific fragments (Table 1), which could be easily differentiated on the basis of size. The primers hybridize to sequences encoding viral E protein, which are considered to be "signature" sequences for each subtype (Fig. 1).

The method was optimized using virus strains belonging to all three TBEV subtypes. The strains were tested for the specificity of the method. In all cases, the procedure led to the subtype-specific bands being easily differentiated with an agarose gel (Fig. 2a). Multiplex RT-PCR revealed that all the strains (166, 263 and 286) isolated in the Czech Republic were members of the European TBEV subtype, which was confirmed by sequence data (Růžek et al., 2006). It was also possible to detect and differentiate the simultaneous presence of TBEV RNA of members of two or all three subtypes in one reaction (Fig. 2b).

Viral RNA was successfully isolated and subsequently amplified in all samples (experimentally infected *I. ricinus* ticks, TBEV-positive mouse organs, human serum samples or suspensions of *I. persulcatus* tick with added TBEV) by multiplex RT-PCR. There was no difference in the sensitivity of this method between samples of different origin.

The sensitivity of TBEV RNA detection was determined for primer sets used individually and in multiplex reactions. Serial 10-fold dilution series were prepared for either the tick homogenates or the serum samples. Using the developed method of multiplex RT-PCR, it was possible to detect viral RNA in samples that had a concentration corresponding to 1-10 PFU/reaction (data not shown). Sensitivity was independent on the sample origin.

Concerning the specificity of the assay, yellow fever virus 17D (a mosquito-borne flavivirus), human herpes simplex virus type 1 and 2 and human enterovirus 71 were assayed with the multiplex RT-PCR reaction. A nonspecific amplification was not observed (data not shown).

4. Discussion

TBEV is of considerable public health importance in various European and Asian countries.

The three subtypes of TBEV – European, Siberian and Far-Eastern – are associated with human infections of different severities. For that reason, rapid differentiation of the viral subtype in areas where co-circulation of members of two or three subtypes occurs is important to determine the appropriate therapeutic procedures.

Recently, it has become very popular to investigate ticks removed from the patient for the presence of TBEV or the spirochete *Borrelia burgdorferi*. Detection of TBEV-positivity in ticks followed by determination of the viral isolate's subtype would give a strong indication to keep the patient under observation for initial symptoms of TBE. This should be accompanied by investigation of repeatedly collected serum samples, whilst undergoing the appropriate therapeutic procedures. Despite the current lack of specific drug therapy for TBE, it is suitable to refine the therapeutic regimen of the patient according to the specific TBEV subtype by which is the patient infected.

The multiplex RT-PCR method that has been developed is capable of the detection and subtyping of TBEV. This method seems to be an excellent tool for detection and simultaneous discrimination of TBEV subtypes in ticks as well as from the material of human origin. This subtyping is based on distinguishing between three possible amplification products of different sizes on agarose gel following RT-PCR amplification.

This method has the potential to save considerable time and effort within the laboratory by avoiding the need for sequence analyses of the E protein encoding sequence of the TBEV isolates. Up until now, sequence analyses or serological investigations were the only methods able to discriminate between the TBEV subtypes. Sequence analysis is expensive and time consuming and serological investigation also has many limitations: cross-reactions with other subtypes or other flaviviruses, requirement of paired samples within periods of 2-3 weeks, possible false positivity with rheumatoid factor, etc. (Steiner et al., 2005).

Several protocols for RT-PCR detection of TBEV RNA in ticks or clinical samples have been described (Ramelov et al., 1993; Whitby et al., 1993; Süss et al., 1997; Schrader et al., 1999; Schweiger et al., 2003; Rudenko et al., 2004). However, none of them are able to discriminate between separate TBEV subtypes. Moreover, quality control assessment study for the PCR diagnosis of TBEV infections revealed that many of the surveyed

laboratories failed to detect TBEV strains of the Siberian and Far Eastern subtype (Donoso Mantke et al., 2007).

PCR detection of TBEV RNA from clinical samples in the early stages of the disease may play an important role in the differential diagnosis of tick-borne encephalitis. Before the appearance of IgM antibodies, viral RNA in serum is detected in almost 100% of cases. However, after the appearance of IgM antibodies, the virus is present only in a low viral load in serum and practically no virus is detectable in samples of cerebrospinal fluid (Saksida et al., 2005). The effectiveness of TBEV detection in serum samples in the early stages of the disease can be also enhanced by the inoculation of the sample into the brains of suckling mice or cultivation of the sample in TBEV susceptible cell line (PS cells, neuroblastoma cells, etc.), that represent a very sensitive environment for the replication of TBE virus, followed by analysis of the brain-homogenate or supernatants of the cell culture for the presence of TBEV RNA.

In conclusion, the method of multiplex RT-PCR has proved to be a very easy and rapid assay that improves the diagnosis of TBE in areas with co-circulation of two or three subtypes significantly facilitating the potential for molecular-epidemiological research of tick-borne encephalitis.

5. Acknowledgment

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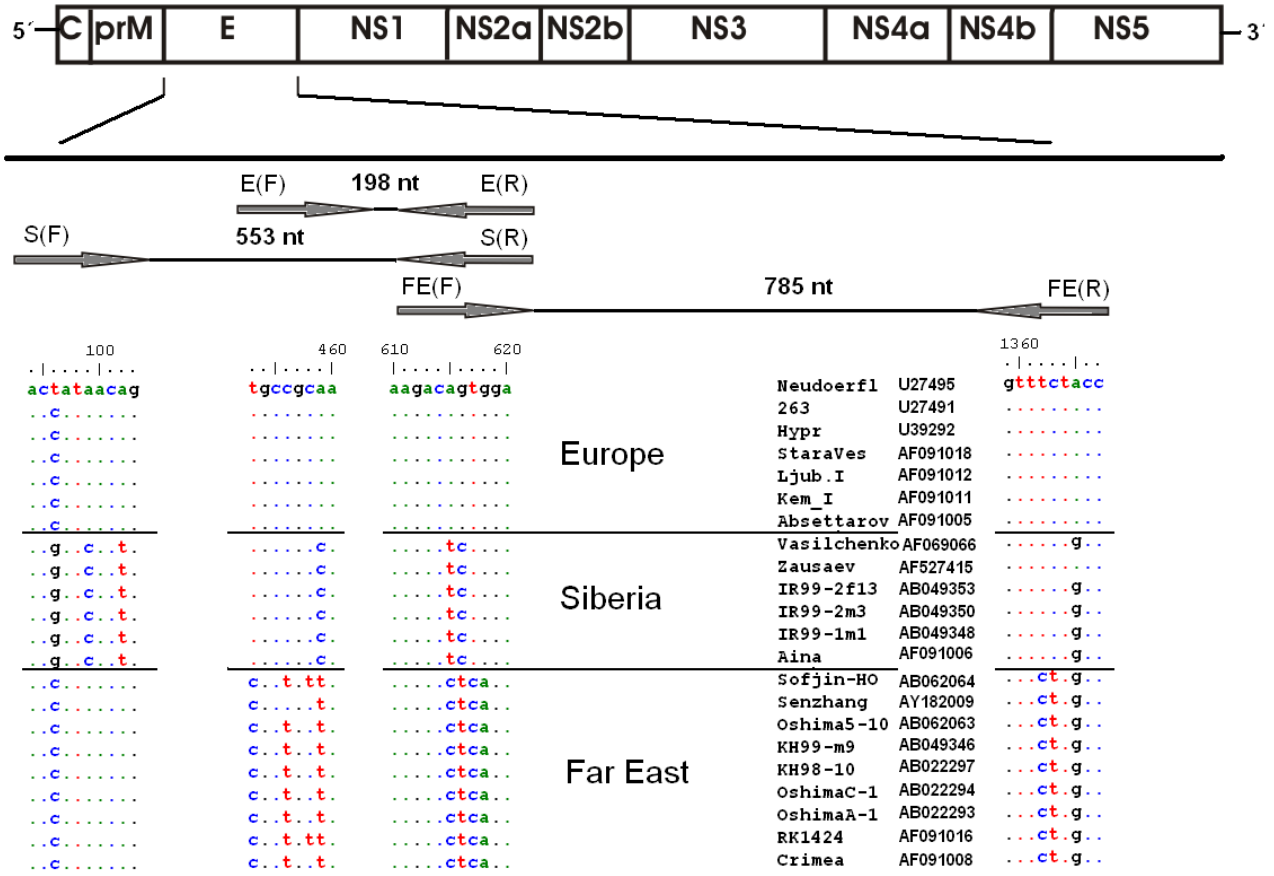

Fig. 1. Schema of the TBEV genome and amplified regions of members of TBEV subtypes. Primers hybridize with subtype-specific „signature“ positions of the sequence encoding viral E protein. The „signature“ positions are demonstrated on the alignment of different members of all three subtypes (numbering according to the nucleotide sequence of E gene of the Europan prototype strain Neudoerfl).

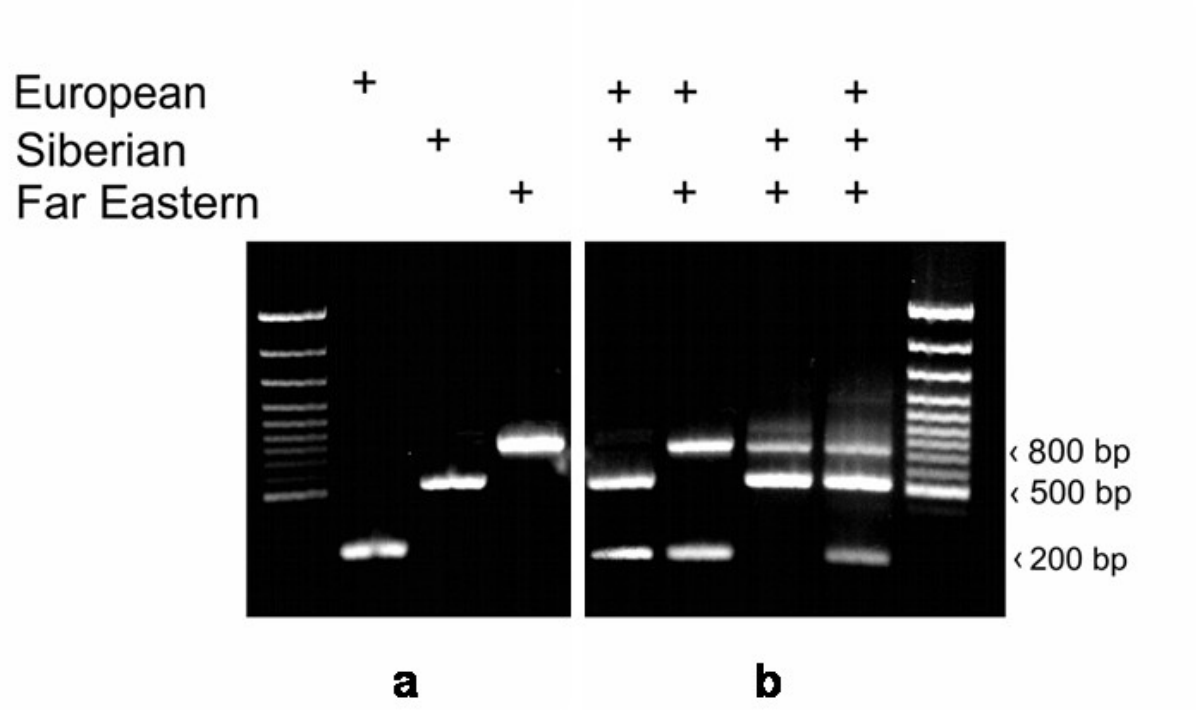

Fig. 2. Subtyping of TBE virus strains using the multiplex RT-PCR. Members of separate subtypes were analyzed individually (a) and in all possible combinations (b).

Table 1

Primers used for subtyping of TBEV isolates by multiplex RT-PCR

Subtype	Primer name	Sequence	Region of amplification[1]	Product length (nts)
European	E(F)	5´-ACA CGG GAG ACT ATG TTG CCG CA-3´	1409-1660	198
	E(R)	5´-CCG TTG GAA GGT GTT CCA CT-3´		
Siberian	S(F)	5´-GKG GAT GTG TCA CGA TCA CT-3´	1057-1601	553
	S(R)	5´-GCY GTY GGA AGG TGT TCC AGA-3´		
Far Eastern	FE(F)	5´-TGG AGC TYG ACA AGA CCT CA-3´	1578-2347	785
	FE(R)	5´-TCC CAC YAG GAT CTT GGG CAA-3´		

[1]Numbering according to the genomic sequence of the prototype strains of TBEV subtypes (European – Neudoerfl, GenBank Acc. No. **U27495**; Siberian – Vasilchenko, **AF069066**; Far Eastern – Sofjin, **X07755**)