Available online at www.sciencedirect.com
ScienceDirect
Journal of Virological Methods 144 (2007) 133–137
Journal of Virological Methods
www.elsevier.com/locate/jviromet

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

Daniel Růžek [a,b,*], Hana Šťastná [b], Jan Kopecký [a,b], Irina Golovljova [c], Libor Grubhoffer [a,b]

[a] Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, CZ-37005 České Budějovice, Czech Republic
[b] Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, CZ-37005 České Budějovice, Czech Republic
[c] Department of Virology, National Institute for Health Development, Hiiu 42, 11619 Tallinn, Estonia

Received 18 November 2006; received in revised form 2 April 2007; accepted 26 April 2007
Available online 4 June 2007

Abstract

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.

Keywords: 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: 3 structural proteins (C, prM, E) and 7 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 three 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); 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 sub-

* Corresponding author. Tel.: +420 38 777 5467; fax: +420 38 531 0388.
E-mail address: ruzekd@paru.cas.cz (D. Růžek).

0166-0934/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2007.04.010

type 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 *Ixodes 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 no. 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 *I. 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 h to feed and then the mice were inoculated subcutaneously with 1000 plaque forming units

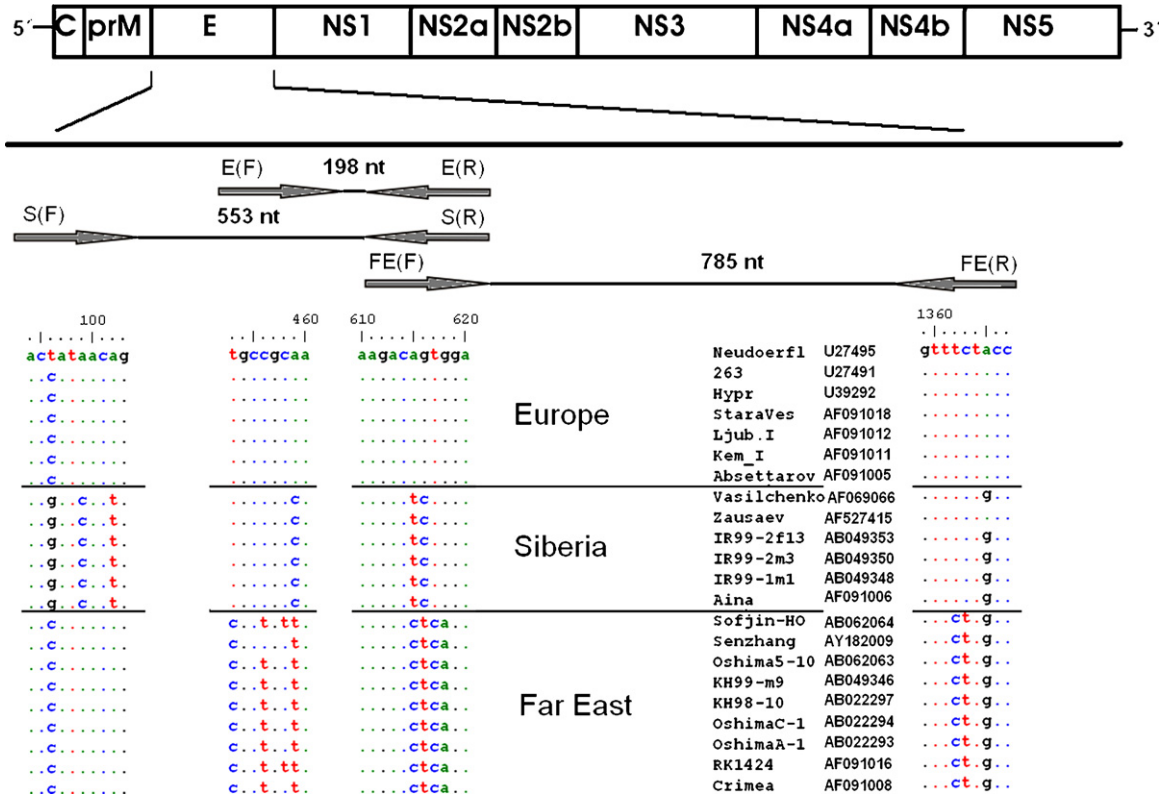

Fig. 1. Schema of the TBEV genome and amplified regions of members of TBEV subtypes. Primers hybridize with subtype-specific "signature" positions of the sequence encoding viral E protein. The "signature" positions are demonstrated on the alignment of different members of all three subtypes (numbering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