TICK-BORNE ENCEPHALITIS VIRUS-SPECIFIC RT-PCR – A RAPID TEST FOR DETECTION OF THE PATHOGEN WITHOUT VIRAL RNA PURIFICATION

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Summary. – Among diseases transmitted by ticks in the Czech Republic, tick-borne encephalitis (TBE) caused by Tick-borne encephalitis virus (TBEV) and Lyme disease caused by *Borrelia burgdorferi* spirochete are most important. We propose an effective and specific test for detection of TBEV in a single tick or a pool of ticks based on the detection of TBEV RNA using an RT-PCR technique without RNA purification. The method is very sensitive with the detection limit of about 14 fg TBEV RNA in total RNA obtained from brain suspension from suckling mice infected with TBEV per reaction. The primers were derived from the 5'-terminal non-coding region, a highly conserved part of the virus. The method was successfully applied to field-collected ticks in detecting TBEV RNA. This method can be used in studies of several aspects of TBEV: epidemiology, screening of natural foci, circulation and detection of virus genome sequences in clinical materials.

Key words: Ixodes ricinus; Tick-borne encephalitis virus; Chelex® 100 resin; RT-PCR; SYBR Green

Introduction

TBEV (the species *Tick-borne encephalitis virus*, the genus Flavivirus, the family *Flaviviridae*) (van Regenmortel *et al*., 2000) is an important human pathogen, endemic in many European countries, Russia and China. TBEV shares common physical and genetic characteristics with other flaviviruses such as Yellow fever virus, Japanese encephalitis virus, Dengue virus and West Nile virus. Based upon geographical origin and antigenic characteristics, the virus has been subdivided into three subtypes: the European subtype, transmitted by *Ixodes ricinus,* includes nearly all isolates from Europe (case fatality rate 0.5–2.0%), the Far Eastern subtype, transmitted by *I. persulcatus,* includes strains from Far Eastern Russia, China and Japan (case mortality rate 5–20%), and the recently described Siberian subtype, transmitted by *I. persulcatus,* that can cause hemorrhagic manifestations in vertebrates (Monath and Heitz, 1996; Gritsun *et al*., 1997; Ecker *et al*., 1999; Mavtchoutko *et al*., 2000; Hayasaka *et al*., 2001; Gritsun *et al*., 2003). The Czech Republic belongs to the countries with the highest rate of TBE infection on the European continent. Current biological methods of TBEV identification in samples are based on antibodies detection by hemagglutination inhibition, neutralization, complement fixation, immunofluorecsence, and enzyme immunoassay. Interpretation of serological tests is complicated by the cross-reactivity between different flaviviruses, delayed immune response, past infections or other factors (Haglund *et al*., 2003). Various PCR and RT-PCR methods for detection of different viruses including TBEV (Labuda *et al*., 1993a, b; Suss *et al*., 1997; Schrader and Suss, 1999) have been described. The success of these methods was dependent on purification of TBEV RNA, its amount and quality. The recently described real-time RT-PCR (Schwaiger and Cassinotti, 2003) can be hardly implemented in a diagnostic

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Abbreviations: FE-TBEV = Far Eastern tick-borne encephalitis virus; nt = nucleotide; S-TBEV = Siberian tick-borne encephalitis virus; TBE = tick-borne encephalitis; TBEV = TBE virus; W-TBEV = Western tick-borne encephalitis virus;

laboratory today because of expensive equipment and supplies.

The aim of this study was to elaborate an improved method of TBEV detection in tick samples using RT-PCR without purification of viral RNA.

Materials and Methods

Virus. The Hypr strain of TBEV was used as a control. The virus material represented a brain suspension from infected suckling mice prepared in Leibowitz medium (L15) supplemented with antibiotics. The titer of the virus in the suspension was 10^8 PFU/ml.

Purification of total RNA. For optimization of reaction conditions and determination of the sensitivity of the method total RNA was isolated and purified from the brain suspension using TRI Reagent (Sigma) according to the manufacturer's instructions. Total RNA represented a mixture of mouse brain RNA and TBEV RNA. The final concentration of purified total RNA was 10 µg/ml.

RT-PCR (control reaction). To detect the presence or absence of viral mRNA transcripts in samples, the Enhanced Avian RT-PCR kit (Sigma) was used. Combination of two powerful and versatile techniques was used to convert mRNA transcripts into cDNA and subsequently to amplify the resulting cDNA. RT-PCR makes possible the detection of very small amount of mRNA from just a few cells. The technique allows conducting one-step (cDNA synthesis and DNA amplification in one tube) or two-step (cDNA synthesis and DNA amplification in two different tubes) RT-PCR reactions. Both possibilities were analyzed to optimize the reaction conditions. The final concentrations of individual components in the RT-PCR reraction mixture (20 µl) were as follows: 10 mmol/l Tris-HCl pH 8.3, 50 mmol/l KCl, 3 mmol/l $MgCl_2$, 0.001% gelatin, 200 µmol/l dNTPs, 0.8 U of RNase inhibitor, 2.5 µmol/l random nonamers, 1 µmole of each PCR primer, 0.5 U of AMV reverse transcriptase, and 0.05 U of AccuTaq DNA polymerase. One µl of purified total RNA was used as a template in control reactions.

Specific primer selection. Two already described primer pairs targeted to different regions of the virus genome were analyzed (Sparagano *et al.,* 1999). The first pair, specific oligonucleotide primers corresponding to the 5'-terminal noncoding region, was successfully used to identify TBEV sequences in ticks: TBE 2 (forward) 5'-GCGTTTGCT(C,T)CGGA-3' and TBE 1 (reverse) 5'-CTCTTTCGACACTCGTCGAGG-3' (Ramelow *et al.*, 1993). The size of the TBE1/TBE2 PCR product was 175 nt. The second pair was derived from the Louping-ill virus sequence at the 3'-terminal region of the E protein gene (nt 19822008 and nt 16001615) and included SH1 (forward, 5'-TTGGCGCT CCTCATG-3') and SH2 (reverse, 5'-TCATAGAGATGCAGCTTCCCCCAGGGG-3') (Shiu *et al.,* 1991). The size of the PCR product was 400 nt.

Annealing temperature. The most critical reaction condition appeared the annealing temperature of the primers. To determine the optimal annealing temperature for primers used a region of 35–55°C with one-degree interval was analyzed. cDNA synthesized from total RNA was used as template. Duration of the denaturation and synthesis steps was constant.

Sensitivity of the reaction. To estimate the sensitivity of the reaction the purified total RNA from brain suspension in concentration of 10 µg/ml was employed. Serial tenfold dilutions were prepared (10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, 100 fg/µl, 10 fg/µl, and 1 fg/µl) and tested in RT-PCR. The products were separated by agarose gel (1.5%) electrophoresis using 1× TBE as running buffer. Gels were stained with SYBR Green Dye (1:10,000 dilution of the stock solution, Molecular Probes, USA)

Direct sequencing of PCR product. To ensure the specificity of the reaction, direct sequencing of the PCR product was performed. The PCR product was cut out of the gel, purified using the QIAquick^O Gel Extraction Kit (Qiagen, Germany) and sequenced twice in both directions with the TBE1/TBE2 primers. The CEQ 2000 Dye Terminator Cycle Sequencing Kit (Beckman Coulter) was used for direct sequencing of PCR product. Sequences were analyzed on the ABI Prism 877 ITC automated DNA sequencer (Beckman). The sequences were analyzed with the DNASTAR software (DNASTAR, Ltd., London, UK) and compared with the data in GenBank. Database searches used the BLAST programs of the National Center for Biotechnology Information (Bethesda, USA).

Tick collection. Nymphs and female adult ticks subjected to RT-PCR analysis were collected by flagging in spring-summer 2002. The collection occurred in two districts in southern Czech Republic with high occurrence of tick-borne encephalitis in humans registered. A hundred *I. ricinus* ticks were analyzed.

Sample preparation from ticks with Chelex® 100 resin. Sample preparation involved no RNA purification. It was sufficient to treat the ticks by Chelex® 100 resin (Bio-Rad Laboratories, USA). This resin bound all possible inhibitors of PCR present in samples. A 5% Chelex 100 resin suspension in TE buffer (10 mmol/l Tris-Cl, pH 7.2, 1 mmol/l EDTA pH 8.0) was found optimal; higher concentration of the slurry did not provide better results. The procedure was as follows: ticks were frozen and then placed individually into 5% Chelex 100 suspension (200 µl per nymph and 500 µl per adult tick). One tick at a time was cut inside the tube using a sharp scalpel. The cutting was performed below liquid level. The scalpel was flamed each time before the next tick was cut. Adult ticks were cut into 4–6 pieces and nymphs were cut into 2–4 pieces. Tubes were vortexed and incubated at 56°C overnight. The samples were then incubated at 98–100°C for 15 mins, vortexed for 10 sec, chilled on ice and centrifuged briefly. The supernatant (up to 12.5 µl) was used as template for RT-PCR. The reaction products were electrophoresed as described above. The sample loading buffer (5×) contained 20% Ficoll, 10 mmol/l Tris-HCl pH 8.0, 1 mg/ml Orange G, 1000x SYBR Green I, 40 mmol/l Tris acetate, and 1 mmol/l EDTA.

Results and Discussion

In the first part of the study the detection of TBEV RNA in total RNA from a brain suspension from TBEV-infected suckling mice by one-step and two-step RT-PCR was tested.

The one-step RT-PCR proved to be more specific, convenient and reliable. Next the starting annealing temperature was optimized.

With the TBE1/TBE2 primers the starting annealing temperature of 35°C gave the expected PCR product with additional non-specific fragments. Increasing this temperature

reduced the amplification of non-specific as well as specific products. The amount of TBEV-specific PCR product amplified at 38°C was much lower than that obtained at 37°C and finally, there was no product at 39°C. The optimal starting annealing temperature for the TBEV RT-PCR was determined as 37°C. Appearance of non-specific products was eliminated by increasing the annealing temperature to 37°C. This temperature was used in the first five cycles of amplification. Following this step, the annealing temperature was increased to 55°C in the remaining 35 amplification cycles to eliminate non-specific amplification in case of Chelex-treated tick samples. Optimal conditions for the one-step RT-PCR were worked out and used as follows: 42°C/45 mins; 95°C/5 mins; 5 cycles of 94°C/30 secs, 37°C/30 secs, and 72°C/1 min; 35 cycles of 94°C/30 secs, 55°C/30 secs, and 72°C/1 min. Two primer pairs were analyzed: TBE1/TBE2 (Ramelow *et al*., 1993) and SH1/SH2 (Shiu *et al*., 1991). The results of these experiments showed that the TBE1/TBE2 primers had the highest specificity. The primers SH1/SH2 did not yield any PCR product at any annealing temperature tested. The gel electrophoresis of the TBE1/TBE2 PCR product from tick samples showed the presence of a single band corresponding to a size a little greater than expected (175 nt) (Fig. 1).

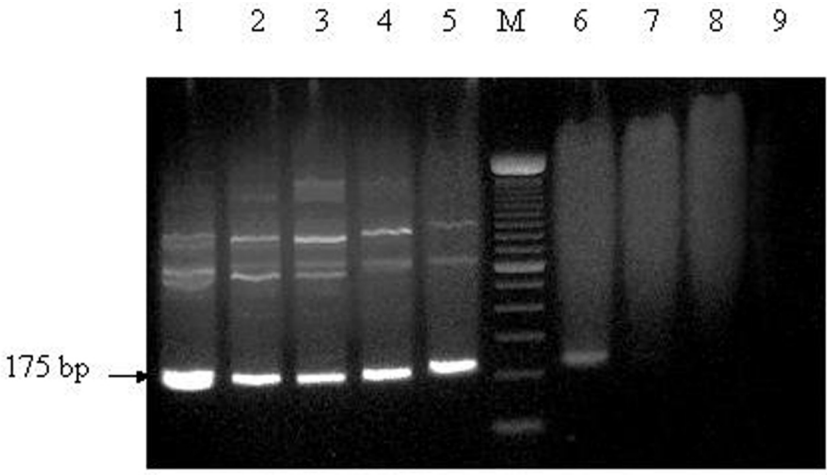

Fig. 1

Estimation of detection limit of TBEV RNA

One-step RT-PCR under optimized conditions. Amounts of TBEV RNA in 1 µl: 10 ng (lane 1), 1 ng (lane 2), 100 pg (lane 3), 10 pg (lane 4), 1 pg (lane 5), 100 fg (lane 6), 10 fg (lane 7), and 1 fg (lane 8). Negative control (lane 9). 100 bp DNA ladder (lane M).

The explanation of this observation came from the manufacturer of SYBR Green Dye Molecular Probes, USA. According to this source the stain has an exceptionally high affinity for dsDNA. In this way its mobility may be affected and the effect is not a linear function of the dsDNA size. The mobility of smaller molecules tends to be affected more that that of larger ones (Molecular Probes, Useful Tips-SYBR Green Nucleic Acid Gel Stains, p.2, 3.2; http://www.probes.com/media/pis/td004.pdf). The amplified TBE1/TBE2 PCR products were cut out of the gel, sequenced in both directions and the obtained sequences were compared to those available in GeneBank. Both appeared to be TBEV-specific 175 nt fragment with 100% homology to the TBEV Hypr strain polyprotein gene (gi|106607|gb|U39292.1|TEU39292).

Next the sensitivity of the RT-PCR (one-step) was determined using total RNA from brain suspension from TBEV-infected suckling mice (for details see Materials and Methods). The number of virus RNA molecules was calculated on the basis of a genome length of 11,141 nt and an average molecular mass of nucleotide of 336.6 g/mol, yielding a mass of 6.2 x 10^{-9} ng per RNA molecule. Using the determined quantitative relationship between PFU and RNA copy (1.3 PFU = 3000 RNA molecules) for the Ross River virus (Sellner *et al*., 1992), we found that the titer of 10^8 PFU/ml corresponds to 1.4 µg of TBEV RNA in the total RNA sample from mice brain. The appearance of TBEV-specific PCR product in the control reaction was safely detected with as little as 100 fg of total RNA from brain suspension of TBEV infected suckling mice (Fig. 1), which corresponds to 14 fg of TBEV RNA per reaction. Our data confirm the already published results on the detection limit of virus RNA. Schrader and Suss (1999) have detected 20 fg of TBEV RNA in a 25 µl of PCR reaction. Sellner *et al.* (1992) were able to detect 18 fg of purified RNA from Ross River virus using an one-tube RT-PCR technique. Sensitive and specific technique with optimized RT-PCR conditions was applied for pathogen detection in ticks from natural foci. Tick samples were prepared by Chelex® 100 resin method that does not demand purification of RNA from tick(s). Viral RNA in such a treated samples is stable at 4°C for at least 6 months. A hundred *I. ricinus* ticks (nymphs and females), collected in the high-risk region of southern Bohemia (Czech Republic), were individually analyzed using the optimized method of one-step RT-PCR. The presence of TBEV RNA transcripts was detected in total five (5%) tick samples. Fig. 2 shows the example of two positive samples. Each experiment was conducted with two controls: total RNA from the brain suspension of TBEV-infected mice (positive control) and TE buffer from the 5% Chelex suspension (negative control). The determined 5% infection rate of the hosts for TBEV corresponds to the data from previous years and indicates the

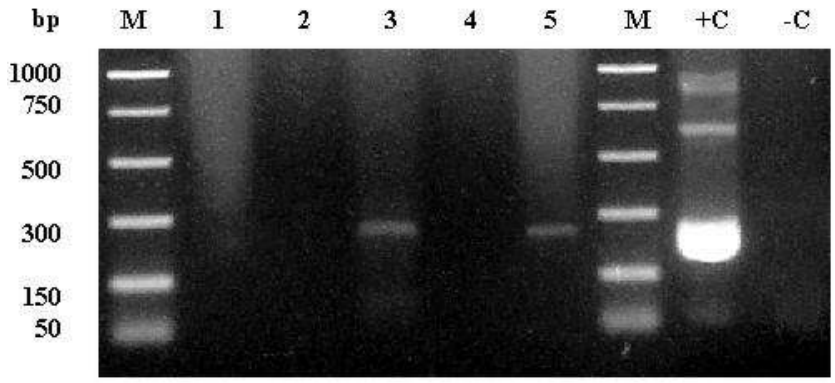

Fig. 2

Detection of TBEV in tick samples

One-step RT-PCR. DNA size markers (Promega, lanes M), tick samples (lanes 1–5), and positive (lane +C) and negative (lane -C) controls.

South Bohemian region of the Czech Republic as an active natural focus with approximately 650–700 cases of TBE per year (1993–2003) (www.szu.cz).

The goal of the present study was to apply a new technique to detect pathogens in a larger number of samples in a short period of time. Many different methods of sample treatment for obtaining better results in PCR or RT-PCR reactions have been already described, but all of them include an RNA purification step. The current PCR methods work usually without toxic reagents (e.g. phenol and chloroform) and with enhanced sensitivity. This feature is of great importance in those cases when the concentration of pathogen in samples is almost as low as the detection limit. However, in spite of all positive features, any purification leads to a sometimes considerable loss of genetic material. Here we describe an efficient system to detect the pathogen in ticks excluding any purification of genetic material from samples. The sensitivity of this method could be compared to that based on RNA purification showing its advantage because RNA isolation/purification is a time and work consuming procedure that can result in serious loss of starting material. The Chelex treatment of clinical samples will make them suitable for the detection of TBEV by RT-PCR.

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