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Protocols

Detection and differentiation of tick-borne encephalitis virus subtypes by a reverse transcription quantitative real-time PCR and pyrosequencing

Katharina Achazi*,[1], Andreas Nitsche[1], Pranav Patel, Aleksandar Radonić, Oliver Donoso Mantke, Matthias Niedrig

Robert Koch Institute, Center for Biological Security 1, Nordufer 20, 13353 Berlin, Germany

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A B S T R A C T

Tick-borne encephalitis (TBE) virus causes one of the most important flaviviral infections of the human central nervous system in Europe and Asia. In recent years the rate of TBE infection has been raising and the virus has been spreading to new areas. Currently, the diagnosis of TBE is based on detection of specific antibodies in patients' sera which appear as late as about 2 weeks post-infection. For a timely diagnosis of TBE virus infections and epidemiological studies, a TBE virus-specific reverse transcription quantitative real-time PCR (RT-qPCR) followed by pyrosequencing was developed. The assay is based on one degenerated primer pair detecting all three human-pathogenic TBE virus subtypes with a detection limit of 10 copies. Even though primers and probe are highly degenerated, the assay is specific for TBE virus species and detects all subtypes with a comparable sensitivity. Furthermore, TBE virus RT-qPCR could be carried out as one-step or two-step assay. RT-qPCR can be followed by pyrosequencing which allows a rapid subtyping of TBE viruses. For detection purposes an internal control to monitor RNA extraction, cDNA synthesis and amplification is included. In summary, the method is sensitive, highly specific and easy-to-handle tool for the detection and differentiation of TBE virus in the early phase of illness or in TBE host animal species and ticks.

1. Introduction

Tick-borne encephalitis (TBE) virus causes one of the most important flaviviral infections of the human central nervous system in Europe and Asia (Thiel, 2005). The TBE virus species belongs to the family *Flaviviridae,* genus *Flavivirus*, along with the etiological agents of diseases such as dengue fever, yellow fever, Japanese encephalitis and St. Louis encephalitis. Like all flaviviruses TBE virus has a linear positive-stranded RNA genome of about 11 kb. This genome consists of a single open reading frame coding for one polyprotein which is cleaved into three structural proteins, E (envelope), C (capsid) and M (membrane), and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (Lindquist and Vapalahti, 2008; Thiel, 2005).

Three subtypes of TBE virus are known: the European, the Siberian and the Far Eastern subtype named according to their main area of circulation. However, there are also areas of co-circulation of the different TBE virus subtypes, e.g. the Baltic States or Finland (Mavtchoutko et al., 2000). According to phylogenetic analysis, these three human-pathogenic TBE virus subtypes are supposed to form the tick-borne encephalitis virus species together with viruses affecting mainly animals such as Louping ill virus (including Spanish, British and Irish subtypes), and Turkish sheep encephalitis virus (including the Greek goat encephalitis virus subtype) (Grard et al., 2007).

Ticks are the main vectors of TBE viruses: *Ixodes persulcatus* in Asia and Eastern Europe and *I. ricinus* in Central, Eastern and Western Europe, respectively (Charrel et al., 2004; Lindquist and Vapalahti, 2008), although TBE virus has also been found in *Dermacentor* spp. in nature and to a far lesser extent in other tick species (Gritsun et al., 2003b).

The TBE virus subtypes are associated with different severity of disease. The European subtype typically causes biphasic illness (Gritsun et al., 2003a). The first phase is characterized by influenza-like symptoms, followed by an asymptomatic interval and a second

Abbreviations: TBE, tick-borne encephalitis; RT-qPCR, reverse transcription quantitative real-time PCR; E, envelope protein; C, capsid protein; M, membrane protein; NS, non-structural protein; DMEM, Dulbecco's modified Eagle medium; MOI, multiplicity of infection; ivRNA, *in vitro*-transcribed RNA; MGB, minor groove binder; FCV, feline calicivirus; C_q, quantitation cycle; MIQE, minimum information for publication of quantitative real-time PCR experiments; bio, biotinylated; LIV, Louping ill virus; SD, standard deviation; LOD, limit of detection.

* Corresponding author. Tel.: +49 30187542917; fax: +49 30187542604.
E-mail addresses: AchaziK@rki.de (K. Achazi), NitscheA@rki.de (A. Nitsche), PatelP@rki.de (P. Patel), RadonicA@rki.de (A. Radonić), DonosoO@rki.de (O.D. Mantke), NiedrigM@rki.de (M. Niedrig).
[1] These authors contributed equally to this work.

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phase when the central nervous system is affected (Charrel et al., 2004; Lindquist and Vapalahti, 2008). In the early stage of the disease, symptoms are non-specific and similar to other tick-borne infections, such as Lyme borreliosis or human granulocytic ehrlichiosis.

In recent years the incidence of TBE diagnosed has increased and TBE virus has been spreading to new areas, e.g. Southern Sweden and Denmark, possibly because of climatic changes (Fomsgaard et al., 2009). Furthermore, the incidence of TBE is increasing in countries where the TBE virus is already common (Donoso Mantke et al., 2008).

Thus, a TBE-specific PCR able to detect all subtypes of TBE virus would greatly benefit differential diagnosis in the early phase of disease (Donoso Mantke et al., 2007b; Saksida et al., 2005; Schultze et al., 2007) despite the fact that virus RNA is only detectable in the early stage of the disease before the development of IgM antibodies or in fatal cases (Gunther et al., 1997; Puchhammer-Stockl et al., 1995). For clinical diagnosis and distribution analysis of TBE virus in samples from patients, host animal species and ticks, a TBE-specific reverse transcription quantitative real-time PCR (RT-qPCR) was developed for detection of TBE virus RNA, followed by pyrosequencing in order to distinguish different subtypes. Fragments obtained from RT-qPCR are difficult to sequence by the Sanger method because fragments have to be as short as possible for a specific and sensitive RT-qPCR assay (Kutyavin et al., 2000). Therefore, pyrosequencing offers the advantage of obtaining sequence data for epidemiological studies and phylogenic analysis from samples found positive by RT-qPCR. An internal control was also included for monitoring RNA extraction, cDNA synthesis and PCR procedures.

2. Materials and methods

2.1. Virus propagation

For test evaluation four TBE virus strains derived from cell culture were used: strains K23 (GenBank accession no. AM600965) and Neudörfl (GenBank accession no. M7779), both European subtypes, strain Aina, Siberian subtype (GenBank accession no. AF091006) and strain Sofjin, Far Eastern subtype (GenBank accession no. X03870) (Heinz et al., 1981; Niedrig et al., 1994; Pletnev et al., 1986; Ternovoi et al., 2003). Vero E6 (ATCC CRL-1586) cells cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 1% glutamine, 1% penicillin and 1% streptomycin were infected with TBE virus (multiplicity of infection [MOI] = 1). After 3–5 days cell culture supernatant was harvested. Viral titers were determined by plaque assay as described by Bae et al. (2003). Non-TBE flavivirus RNA and non-flavivirus RNA as well as DNA samples for the specificity test were obtained from the Robert Koch Institute reference stocks (Table 1).

Table 1
Results obtained in the specificity tests for TBE virus RT-qPCR assay.[a]

Virus sample	Result of RT-qPCR
TBE virus European subtype; strains: K23, Neudörfl, Absettarov, Petracova, Trypsorski, Klodobok, 274 II, Gbelce and Dobrostan[b]	Positive
TBE virus Siberian subtype; strains: Aina and 179-78[b]	Positive
TBE virus Far eastern subtype; strains: Sofjin and RSSE[b]	Positive
Louping ill virus[b]	Positive
Coxsackie virus type B5[b]	Negative
Echo virus types 30 and 2[b]	Negative
Measles virus strains C2, D7 and D6[b]	Negative
Herpes simplex viruses 1 and 2[c]	Negative
Yellow fever virus strain 17D[b]	Negative
Japanese encephalitis virus[b]	Negative
West Nile virus strains New York and Kunjin[b]	Negative
Dengue viruses 1, 2, 3 and 4[b]	Negative
Saint Louis encephalitis virus[b]	Negative

[a] Samples were measured in duplicate in TBE RT-qPCR.
[b] Nucleic acids were isolated from supernatant of infected cell cultures containing at least 10^4 viral copies per ml.
[c] Nucleic acids were isolated from patients, containing at least 10^3 viral copies per ml.

2.2. Isolation of viral nucleic acids

Total nucleic acids from cell culture supernatants, human serum or cerebrospinal fluid samples were extracted using the QIAamp Viral Mini Kit or the QIAamp DNA Mini Kit, respectively (Qiagen, Hilden, Germany, http://www.qiagen.com), according to the manufacturer's instructions.

Rodent brain or spleen tissue samples from trapped wild rodents were first homogenized with Fast-Prep FP120 (eubio, Vienna, Austria, http://www.eubio.at) in a reaction tube (Sarstedt, Nümbrecht, Germany, http://www.sarstedt.com) with about 10 ceramic beads 1.4 mm (PeqLab, Fürth, Germany, http://www.peqlab.de). In a second step, RNA was extracted from supernatant obtained after centrifugation of the sample (16,000 × *g*, 5 min, 4 °C), according to the protocol described by Chomczynski and Sacchi (1987). The trapping and use of rodents was approved by an Ethics Committee under established animal welfare conditions.

RNA from laboratory-bred *I. ricinus* ticks was extracted with the rapidStripe Tick DNA/RNA Extraction Kit (Analytic Jena AG, Jena, Germany, http://www.analytik-jena.de) according to the manufacturer's instructions. Each tick was processed separately.

Sera and cerebrospinal fluid were stored at 4 °C for a maximum of 1 day before extracting RNA. Rodent brain tissue samples, ticks and cell culture supernatants were frozen at −80 °C directly after dissection or sampling, respectively, and were thawed for RNA extraction. RNA was stored at −80 °C until processing (4 weeks at most).

2.3. Reverse transcription, plasmid cloning and in vitro RNA standards

To obtain cDNA for the two-step reaction and the cloning of standard plasmids containing TBE virus-specific target sequences, 5 μl of sample RNA were reverse transcribed in a 20 μl final reaction volume with Superscript II and random hexamer primers (Invitrogen, Karlsruhe, Germany, http://www.invitrogen.com). RNA was pre-treated for 10 min at 65 °C. Temperature parameters were set to 60 min at 37 °C and 10 min at 93 °C on a Biometra thermoblock cycler (Biometra, Göttingen, Germany, http://www.biometra.de).

To test subtype specificity of the PCR, cDNAs from European subtype K23, Siberian subtype Aina and Far Eastern subtype Sofjin were amplified using the primer pair NS1 clone F and R (NS1 clone F: gAgATggCCATgTggAgRA, nt 2650–2666; NS1 clone R: gTACTTCCATggYCCTTTCA'; nt 3273–3254; GenBank accession no. U27495). The amplicons were cloned into a pDrive cloning vector (Qiagen) according to the manufacturer's instructions. Plasmid copy number was determined by measuring plasmid DNA concentration by BioPhotometer (Eppendorf, Hamburg, Germany, http://www.eppendorf.de) at 260 nm, and insert orientation was checked by sequencing.

In vitro-transcribed RNA (ivRNA) was synthesized by the Riboprobe *in vitro*-Transcription system T7 (Promega, Mannheim, Germany, http://www.promega.com), using the pDrive vector containing cDNA of K23 amplified with the primers NS1 clone F and R. A DNase digest to remove contaminating DNA from the ivRNA was done by TURBO DNA-free Kit (Applied Biosystems/Ambion, Foster City, USA, http://www.appliedbiosystems.com) according to the