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First detection of tick-borne encephalitis virus RNA in clinical specimens of acutely ill patients in Hungary

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ABSTRACT

Tick-borne encephalitis virus (TBEV) is one of the endemic flaviviruses in Hungary, which is responsible for human infections every year. Neurological involvement in the disease is characterized by meningitis, encephalitis or meningoencephalitis which can result in long-term neurological and neuropsychiatric sequelae. Microbiological diagnosis of acute cases is predominantly based on serological tests due to the limited duration of viremia and long incubation period, however, the application of molecular methods can also supplement the serological diagnosis and provides epidemiological data. The aim of this study was to determine how viral RNA could successfully be detected from different body fluids of serologically confirmed acute cases. Serum, whole blood, cerebrospinal fluid and urine samples of 18 patients from the total of the 19 serologically diagnosed cases were investigated by using the RT-PCR method. Two sera and one urine sample of three patients tested positive and the European subtype of TBEV could be identified. As far as we know this was the first time that TBEV RNA could be detected from human clinical samples in Hungary. Our finding highlights that the application of molecular methods besides serological tests can be a valuable tool in differential diagnosis especially in areas like Hungary, where two or more flaviviruses are co-circulating.

1. Introduction

Tick-borne encephalitis virus (TBEV) is one of the most important endemic viral zoonoses in Hungary and the causative agent of human infections every year. TBEV belongs to the *Flavivirus* genus of the family *Flaviviridae* and has three antigenically closely related subtypes: European, Siberian, and Far Eastern (Mansfield et al., 2009; Donoso Mantke et al., 2008). The main transmission route for the European-subtype is via the bite of *Ixodes ricinus* ticks, however, less frequently infections can occur by consumption of infected unpasteurised milk or dairy products (Balogh et al., 2010; Caini et al., 2012). The majority of human infections are asymptomatic, while most of the clinically manifested cases have a biphasic course.

The incubation period ranges from 7 to 14 days, followed by a febrile, viremic phase characterized by nonspecific symptoms including fever, fatigue, headache, and myalgias. After a short symptom-free period, central nervous system involvement is marked by high fever, meningitis, meningoencephalitis, or meningoencephalomyelitis (Růžek et al., 2010; Haglund et al., 2003). In severe cases, patients can experience unconsciousness and/or poliomyelitis-like syndrome. The case mortality rate is less than 2% in Europe (Donoso Mantke et al., 2008).

Depending on the causative subtype there are some differences in the severity of the resulting central nervous system disorders and clinical outcomes (Růžek et al., 2010; Gritsun et al., 2003).

TBEV is a positive, single-stranded RNA virus and the genome is approximately 11 kilobases in length containing a single open reading frame which encodes three structural (C, M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Mansfield et al., 2009; Gritsun et al., 2003). The 5′ and 3′ termini of the genome contain non-coding, untranslated sequences, which are sufficiently conservative PCR targets for detection of the different TBEV strains (Mansfield et al., 2009). Clinical and laboratory case definition criteria for reporting tick-borne encephalitis (TBE) cases are applied using the European Union's guidance. Any person with symptoms of the inflammation of the central nervous system meets the clinical criterion of TBE, while for laboratory case confirmation at least one of the following five criteria must be met (European Commission Implementing Decision, Brussels, 2012):

- TBEV-specific IgM and IgG antibodies in blood;
- TBEV-specific IgM antibodies in cerebrospinal fluid (CSF);
- Seroconversion or four-fold increase of TBEV-specific antibodies in

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paired sera;
- Detection of TBEV nucleic acid in the clinical specimen;
- Isolation of TBEV from the clinical specimen.

Common laboratory criterion for a probable TBE case is the detection of TBEV-specific IgM antibodies in a unique serum sample (European Commission Implementing Decision, Brussels, 2012).

Laboratory diagnosis is primarily based on serological tests designed to detect virus-specific antibodies only, due to the brief viremic phase. Most of the patients are admitted to hospital during the non-viremic second-phase at the time of onset of neurological symptoms, when the virus is usually no longer detectable (Růžek et al., 2010; Formanová et al., 2014). There are some factors which could complicate the serological diagnosis: patients infected with other flaviviruses produce antibodies cross-reacting with serological tests for detection of TBEV-specific humoral immune response. Besides the serological cross-reactions, the long-term persistence of IgM antibodies in serum and the phenomenon of original antigenic sin in case of secondary flavivirus infection could significantly complicate the serological diagnosis. Therefore, detection of viral nucleic acid could be an efficient method for differential diagnosis of acute infections especially in areas like Hungary, where two or more flaviviruses co-circulate. A few studies have already described the possibility of viral RNA detection in serum, whole blood, CSF, or urine samples, and found that the virus can be most efficiently demonstrated in first-phase serum or whole blood and occasionally in urine (Haglund et al., 2003; Caracciolo et al., 2015; Puchhammer-Stöckl et al., 1995; Saksida et al., 2005; Veje et al., 2014). The aim of this study was to investigate different body fluids (serum, whole blood, CSF, and urine) from acutely ill patients by molecular methods and to examine how successfully TBEV RNA can be detected in these clinical specimens. Additionally, PCR-positive samples were further tested in order to identify the TBEV strains that cause human infections in Hungary.

2. Material and methods

Serological investigation of clinically suspected TBE cases were primarily carried out by indirect immunofluorescent assay (IFA) and ELISA tests. Results were confirmed by an in-house haemagglutination-inhibition (HI) test. Primarily, human serum and CSF samples were examined for virus-specific IgG, IgM and/or IgA antibodies by in-house immunofluorescent assay which method is validated by external quality assurance tests. In case of IgG positive reaction, IgM/IgA response and IgG antibody

end-point titers were determined, and the results were confirmed by an in-house haemagglutination-inhibition test. Besides the in-house serological assays, a commercially available ELISA test (FSME/TBE IgG/IgM ELISA; Sekisui Virotech GmbH) was also used to strengthen the IgM and IgG results. In order to rule out the presence of cross-reactive antibodies, serological investigation of other flaviviruses circulating in Hungary – such as West Nile virus – was performed in parallel. All laboratory results were interpreted according to the patient's flavivirus vaccination status.

Serum, whole blood, CSF, and urine samples from serologically confirmed and suspected acute cases were further examined by molecular methods.

Viral nucleic acid was extracted from 140 µl of specimens using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden) according to the manufacturer's instructions. Whole blood was first diluted in UltraPure RNase/DNase-free distilled water (Gibco): 50 µl whole blood was added to 90 µl PCR grade water. Viral RNA was eluted from QIAamp spin columns in 60 µl final volume and was stored at −80 °C until further use.

5 µl of viral RNA was reverse transcribed according to the following protocol: 1 µl of UltraPureDNase/RNase-free distilled water (Gibco), 2 µl of GeneAmp® 10X PCR Buffer (Invitrogen), 4 µl of 25 mM $MgCl_2$ (Thermo Scientific), 8 µl of 10 mMdNTPmix (Applied Biosystems), 1 µl of 50 µM random hexamer primers (Invitrogen), 1 µl of 50U/µl of MuLV Reverse Transcriptase (Invitrogen) and 1 µl of 20U/µl RNase Inhibitor (Invitrogen) were mixed. Reverse transcription was incubated at 42 °C for 30 min, followed by enzyme inactivation at 99 °C for 5 min and rapid cooling to 4 °C.

cDNA was analyzed by TaqMan real-time PCR assay using primers and probe targeted to the part of the 3′non-coding region of the viral genome (Brinkley et al., 2008).The fluorogenic hydrolysis probe was labelled with 5′ FAM (6-carboxyfluorescein) and 3′ TAMRA (6-carboxytetramethylrhodamine). The reaction mixture consisted of the following reagents in 20 µl final volume: 10 µl of template cDNA, 3.5 µl of UltraPure RNase/DNase-free distilled water (Roche Life Science), 4 µl of ready-to-use hot start master solution (LightCycler®TaqMan®Master, Roche Life Science), 1 µM of each primer and 0.125 µM of the probe. Amplification started with initial denaturation and hot start polymerase activation: 95 °C for 10 min followed by 45 PCR cycles (95 °C 15 s, 60 °C 45 s, 72 °C 15 s). The reaction was carried out in Light Cycler 2.0 instrument (Roche Life Science).

In case of weak positive or indeterminate results, when cycle threshold (Ct) value is higher than 35.00, the real-time assay was verified by nested PCR. Two sets of primers specific to the capsid and membrane overlapping region were designed following general primer design rules: KEVf1 (CTTAGGAGAACAAGAGCTGGG) and KEVr1 (TCATCACACCATGACCCCAT) for first round amplification and KEVf2 (CGACGAGTGTCGAAAGAGACCG) and KEVr2 (GCCAGGATCACAC AGGTGCCAT) for nested PCR. The consistency of the first-round reaction mixture was: 5 µl of template cDNA, 10 µl of UltraPure DNase/RNase-free distilled water (Gibco), 25 µl of 2X MyTaq™Red Mix (Bioline), and 1.25 µM of each primer in 50 µl final volume. Nested PCR mixture contained: 4 µl of first-round PCR product, 16 µl of UltraPure DNase/RNase-free distilled water (Gibco), 25 µl of 2X MyTaq™Red Mix (Bioline), and 1 µM of each primer in 50 µl final volume.

PCR amplicons were visualized in a 2% Tris-borate-EDTA agarose gel stained with ethidium bromide.

Nested PCR products were purified using PCR Advanced™PCR Clean UpMiniprep System (ViogenBiotek Corporation) following the manufacturer's instructions. Direct sequencing of the amplicons was performed on 3500 Genetic Analyzer (Applied Biosystems) using BigDye® Terminator V3.1 cycle sequencing kit (Applied Biosystems). Nucleotide sequences were identified using the Basic Local Alignment Search Tool (BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic neighbour-joining tree was constructed with the MEGA 5.05 (Molecular Evolutionary Genetic Analysis) software generating a thousand replicates for bootstrap testing and using a Kimura 2-parameter model for calculating evolutionary distance.

3. Results

The total number of the serologically investigated clinically ill patients was 508 in 2016. During the seasonal period 19 acute TBE cases were diagnosed by the Hungarian National Reference Laboratory for Viral Zoonoses: 14 patients met the laboratory confirmed case definition criteria, whilst five cases were defined as suspected acute infections. Totally, 24 sera, five whole blood, 13 CSF and 15 urine samples were available from the 19 patients. From five patients, paired sera were also sent to the laboratory to verify the serological results. Seroconversion was observed in only one patient (Hun No. 3, Table 1). Furthermore, in case of one person, only serological tests were performed because of the inadequate storage of the serum and CSF samples and the long time period elapsed between the onset of symptoms and the date of sampling.

Twenty sera (18 sera from the 19 patients and two additional paired sera from patient Hun No. 1; Hun No. 3, Table 1), five whole blood, 12 CSF and 15 urine samples were examined by molecular methods. TBEV-specific real-time PCR showed weak positive or inadequate (Ct >