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Original Article

Hungarian tick-borne encephalitis viruses isolated from a 0.5-ha focus are closely related to Finnish strains

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ABSTRACT

Four tick-borne encephalitis virus strains were isolated from a small 0.5-ha focus over a six-year-long period (2011–2016) in Hungary. Two strains with identical genomes were isolated from *Ixodes ricinus* and *Haemaphysalis concinna* two months apart, which shows that the virus had not evolved separately in these tick species. Whole-genome sequencing of the virus revealed that the isolates differed from each other in 4 amino acids and 9 nucleotides. The calculated substitution rates indicated that the speed of genome evolution differs from habitat to habitat, and continuously changes even within the same focus. The amino acid changes affected the capsid, envelope, NS2a and NS5 genes, and one mutation each occurred in the 5′ and 3′ NCR as well as the premembrane, NS2a and NS5 genes. Phylogenetic analyses based on complete coding ORF sequences showed that the isolates belong to the European subtype of the virus and are closely related to the Finnish Kumlinge strains, the Bavarian isolate Leila and two isolates of Russian origin, but more distantly related to viruses from the neighbouring Central European countries. These isolates obviously have a common origin and are probably connected by migrating birds. These are the first published complete Hungarian TBEV sequences.

1. Introduction

Tick-borne encephalitis is a well-known vector-borne viral disease, one of the most important zoonoses in Europe (Mansfield et al., 2009; Süss, 2011). *Tick-borne encephalitis virus* (TBEV) is member of the genus *Flavivirus*. The virus exists in nature in a complex cycle influenced by tick and small mammal populations, weather, climate, precipitation, and vegetation cover (Haemig et al., 2011). The virus is mostly transmitted to humans by ticks, but infections via the consumption of contaminated unpasteurised raw milk of ruminants has also been reported (Balogh et al., 2010; Daniel et al., 2011). The pathogenicity of the virus in humans depends on species, age, and individual susceptibility (Balogh et al., 2010; Bogovic et al., 2010; Ružek et al., 2010; Kaiser, 2012). The disease can be prevented by the use of commercially available formalin-inactivated vaccines (Heinz et al., 2007), which effectively decreased the diagnosed TBEV cases in Austria and Hungary.

Phylogenetic studies distinguished three main subtypes of TBEV, the European (TBEV-Eur), the Siberian (TBEV-Sib) and the Far-Eastern (TBEV-FE) subtypes (Ecker et al., 1999). The first is mostly transmitted by *Ixodes ricinus* while the other two by *Ixodes persulcatus,* although *I. ricinus* is able to carry Siberian and *I. persulcatus* the Western subtype (Jääskeläinen et al., 2016). Severe neurological symptoms are more frequent with the Far-Eastern subtype, while Siberian strains usually cause chronic disease, but both subtypes have been associated with chronic and progressive disease (Gritsun et al., 2003). The Western subtype causes a less severe, benign type of infection with flue-like start, with biphasic fever, later meningitis and encephalitis (Kaiser 2012). Comparing clinical symptoms of 100 human TBEV cases from 1976 to 1986 to 93 cases from 1987 to 1996 12 of the 18 studied symptoms showed statistically significant softening, which indicates circulation of less virulent strains or more resistant human population in Hungary (Lakos et al., 1996–1997).

Antigen stability of natural TBEV isolates was established by studying envelope proteins of seven TBEV strains isolated from three Austrian foci 14 years apart, using a panel of 14 monoclonal antibodies (Guirakhoo et al., 1987). The tests revealed no considerable difference among the strains either by serology or by electrophoresis. The mutation rate of the TBEV genome is much lower than that of other RNA viruses such as HIV (Li et al., 1988; Uzcategui et al., 2012).

The tick-borne encephalitic flaviviruses appear to have evolved in Africa, gradually from non-encephalitic viruses that radiated Asia. Epidemiological studies outline various theories about spread, divergence of TBEV strains from Middle East to the Far-East, Russia and Western Europe during the past two to four thousand years (Gould

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et al., 2004; Uzcategui et al., 2012; Heinze et al., 2012).

TBEV was also isolated (Brummer-Korvenkontio et al., 1973), and detected by enzyme immunoassay and RT-PCR from wild birds (Mikryukova et al., 2014) and identified from ticks found on migratory birds (Waldenström et al., 2007). 7% of birds arriving in Norway and 3% of birds in Sweden carried ticks (Waldenström et al., 2007; Hasle et al., 2009).

From the 1950s the virus annually caused 200–300 human cases in Hungary (Molnár and Kubasova, 1966–1967). Over the subsequent three decades dozens of strains were isolated (Fornosi and Molnár, 1954). Unfortunately, the viability of these strains was lost because they had been kept frozen for decades, which prevented the sequencing of full genomes. The only TBEV sequence available online from Hungary so far is a partial sequence of the envelope gene (1488 nt AF091011) of strain KEM-1 (Ecker et al., 1999).

The aim of the present study was to isolate new TBEV strain(s) exploiting an unprecedentedly small (0.5-ha) known natural focus, in order to sequence the viral RNA, perform phylogenetic analyses and study the natural evolution of the TBE genome, thus filling the gap left by the missing Hungarian data in European TBEV epidemiological studies.

2. Materials and methods

2.1. Field work, tick collection, isolation

Ticks were collected from a pasture where a milk-borne TBEV epidemic had broken out in 2007 (Balogh et al., 2010). The infected area was localized to a 0.5-ha area where ticks were sampled regularly at monthly visits (from April to October) for eight years from 2010 to 2017 (Zöldi et al., 2015). For the first five years ticks were sampled from the vegetation by the drag-flag method, while in the last three years they were collected from live-captured small rodents. Rodent specimens were thoroughly examined for infested ticks with a 12X magnifier glass. The observed ticks were removed from the rodents by Rubis-7 SA tweezers (Outils Rubis SA, Stabio, Switzerland), then the small mammals were released alive at the exact sites of their capture. Ticks were collected in 1.5-ml Eppendorf tubes. The tubes were cooled and were kept at 4 °C in a cooling box until transported to the laboratory. The tick species were separated, 10 nymphs and 50 larvae were pooled as one sample. The ticks were smashed in sterile mortars and suspended in 400 µl DMEM, 20 µl of which were inoculated intracranially to suckling NMRI laboratory mice. The permission for this animal study was granted by the National Food Chain Safety Office (permission no. PEI/001/1792-4/2014), and the study was undertaken under the supervision of the local animal protection committee at the Veterinary Medical Research Institute.

2.2. RNA extraction, cDNA synthesis, PCR assays

RNA was extracted from 30 µg of brain tissue (homogenised in sterile mortars) using a Nucleospin RNA Plus kit (Macherey-Nagel GmbH, Düren, Germany) following the manufacturer's instructions. Reverse transcription was performed using AMV reverse transcriptase system (PROMEGA Madison, WI, USA) according to the manufacturer's protocol. Seven nested PCRs (Asghar et al., 2014) were performed in order to obtain overlapping fragments covering the entire genome using Quantitect Probe PCR kit (Qiagen, Hilden, Germany).

The resulting fragments were purified, mixed in an equimolar manner and sequenced on an Illumina platform (Illumina Inc.) by Xenovea Company (Szeged, Hungary). The obtained consensus Fasta files were checked, reanalysed and edited if needed, using the generated Illumina FastQ files with the software included in the DNASTAR Lasergene package version 15 (Madison, Wisconsin USA).

The genome sequences of the four strains were deposited in the GenBank under the accession numbers KEM118-MG210945, KEM125-MG210946, KEM127-MG210947 and KEM168-MG210948.

2.3. Sequencing, phylogenetic analysis

Phylogenetic analysis of the four Hungarian strains and 39 TBEV-Eur sequences available in the GenBank was performed on nucleotide sequences of the complete polyprotein coding region. The Russian strain Primorye 332 (TBEV-FE subtype GenBank accession number: AY169390) was used as outgroup. Sequences were aligned using the web-based multiple alignment software MAFFT version 7 (Katoh et al., 2017). Phylogenetic analysis was inferred by using the Maximum Likelihood method in MEGA 6 (Tamura et al., 2013) based on the General Time Reversible model with 4 Gamma categories with Invariant sites (Nei and Kumar, 2000) and 1000 bootstrap replicates. The model was chosen on the basis of the statistical selection results of best-fit models obtained with the software jMODELTEST2 (Darriba et al., 2012).

3. Results

3.1. Strain isolations, detection of mutations

Although the field work described above lasted for eight years (2010–2017), only four isolates were found in the 2011–2016 period, one in 2011 (KEM-118), two in 2012 (KEM-125 and KEM-127), and one in 2016 (KEM-168); for detailed data see Table 1. A total of 10,994 nucleotides of the genome of our isolates were determined and their sequence data were deposited in GenBank (accession numbers: MG210495, MG210496, MG210497, MG210498).

Compared to the strain isolated in 2011 (KEM-118), four mutations were found in the strains isolated nine and eleven months later (KEM-125 and KEM-127) and in addition to these nucleotide changes, five more were detected in the strain isolated in 2016 (KEM-168). The complete genome sequences of KEM-125 and KEM-127 isolated in 2012 were identical.

Out of the nine point mutations that the sequencing revealed between our first and last isolates (118/168), four were sense mutations of the coding region, i.e. resulted in amino acid (aa) changes (see Table 2). No TBEV sequences deposited in the GenBank had these mutations except the substitution of T-C at position 40 of the 5′ non-coding region (NCR) in KEM-168, which was also found in the Komi 10-09 sequence from the north-eastern corner of European Russia near the Ural mountains (JX628801).

The four sense mutations affected the capsid, envelope, NS2a and NS5 genes, while the five substitutions were identified in the premembrane, envelope and NS5 genes, and at the 5′ and 3′ NCR. The 3′-NCR region of all isolates was 620 nt long, without deletions and polyA tails.

Isolates from 2011 and 2012 differed in four substitutions, from 2012 to 2016 five more nucleotide changes developed, so the final difference between the first (2011) and the last (2016) isolate was nine

Table 1
Data of the sequenced TBEV strains, isolated from the same 0.5-ha focus over six years.

Strains	Date of isolation	Tick species	Developmental stage	Source of ticks	Genbank Accession No.
KEM-118	2011 August	*Ixodes ricinus*	larva	vegetation	MG210945
KEM-125	2012 May	*Ixodes ricinus*	nymph	vegetation	MG210946
KEM-127	2012 July	*Haemaphys. concinna*	nymph	vegetation	MG210947
KEM-168	2016 August	*Ixodes ricinus*	larva	host (*A. agrarius*)	MG210948