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Research paper

New genetic lineage within the Siberian subtype of tick-borne encephalitis virus found in Western Siberia, Russia

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ABSTRACT

Tick-borne encephalitis virus (TBEV), a member of the Flaviviridae family, is a causative agent of a severe neurological disease. There are three main TBEV subtypes: the European (TBEV-Eu), Far Eastern (TBEV-FE), and Siberian (TBEV-Sib). Currently, three lineages within TBEV-Sib have been recorded. In this study, the genetic and biological characteristics of a new original strain, TBEV-2871, isolated in the Novosibirsk province of Western Siberia, Russia were investigated. The strain has low neuroinvasiveness in mice. Phylogenetic analysis demonstrated that TBEV-2871 belongs to TBEV-Sib, but does not cluster with any of the TBEV-Sib lineages. The TBEV-2871 strain has 88–89% nucleotide sequence identity with the other TBEV-Sib strains, 84–86% nucleotide sequence identity with the TBEV-FE and TBEV-Eu subtypes and is genetically close to the subtype division border. The TBEV-2871 polyprotein sequence includes 43 unique amino acid substitutions, 30 of which are recorded at positions that are conserved among all TBEV subtypes. Strain TBEV-2871 and two similar but not identical isolates found in Kemerovo province, Western Siberia are separated into a new lineage tentatively named Obskaya after the name of Ob riber, in the vicinity of which the TBEV-2871 was first found. A molecular evolution investigation demonstrated that within TBEV-Sib, the Obskaya lineage likely separated 1535 years ago, which is even earlier than the Baltic lineage.

1. Introduction

Tick-borne encephalitis virus (TBEV), a member of the genus *Flavivirus* (family Flaviviridae), is a causative agent of human disease of the central nervous system, tick-borne encephalitis (TBE). Clinical manifestations of this infection vary from asymptomatic and mild forms to meningoencephalomyelitis with severe complications and even a fatal outcome (Donoso Mantke et al., 2008). TBEV is an enveloped virus with a positive-sense single-stranded RNA genome approximately 10.5 kb in length which includes a 5′ type I cap, 5′- and 3′-noncoding regions and one long open reading frame (ORF). This ORF encodes three structural (C, prM, and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Knipe and Howley, 2013).

Based on sequence analysis of the E gene and later, the complete genomes, three main TBEV subtypes have been classified: the European (TBEV-Eu), the Far Eastern (TBEV-FE), and the Siberian (TBEV-Sib) subtypes (Gritsun et al., 1993; Ecker et al., 1999; King et al., 2012). Within each subtype, different genetic lineages could be identified. Currently, several TBEV-Eu lineages have been described by different researchers (Lommano et al., 2012; Uzcátegui et al., 2012; Rieille et al., 2014; Demina et al., 2017): at least four separate lineages have been found within TBEV-FE (Hayasaka et al., 1999; Subbotina and Loktev, 2012; Zlobin et al., 2001a, 2001b), and three lineages within TBEV-Sib (Gritsun et al., 1993, 2003a; Zlobin et al., 2001a, 2001b; Golovljova et al., 2004, 2008; Tkachev et al., 2011; Tonteri et al., 2011). The diversity of the E protein between strains within the subtypes is low, about 1.2–2.2% at the amino acid level, while a difference of 5.6% has been found between the three subtypes, which is in the range of variation reported for other flaviviruses (Ecker et al., 1999).

It was demonstrated previously that TBEV-Sib is a dominant subtype in ticks collected in the suburbs of Novosibirsk city, Western Siberia of Russia (Tkachev et al., 2011; Bakhvalova et al., 2016; Rar et al., 2017). Here, three TBEV-Sib lineages have been described: Zausaev and Vasilchenko lineages, named after the prototype strains (Zausaev, AF527415, and Vasilchenko, AF069066) found in Siberia, Russia (Gritsun et al., 1993, 2003a; Zlobin et al., 2001a, 2001b; Tkachev et al.,

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2011), and the Baltic lineage detected at first in Baltic countries and in the European part of Russia (Golovljova et al., 2004, 2008; Khasnatinov et al., 2009; Tonteri et al., 2011). TBEV-Sib is believed to cause a less severe disease compared to TBEV-FE with a tendency for patients to develop chronic infections accompanied by diverse neurological and/or neuropsychiatric symptoms more often than TBEV-Eu (Gritsun et al., 2003b; Donoso Mantke et al., 2008; Charrel et al., 2004; Poponnikova, 2006; Lindquist and Vapalahti, 2008; Mansfield et al., 2009). However, patients with different forms of TBE varying from mild infection to severe forms including cases with fatal outcome have been recorded in Novosibirsk city every year. In addition to differences in the immune status of a patient, TBEV genetic peculiarities can influence clinical manifestations of the disease. Here, we describe an unusual TBEV strain isolated in the Novosibirsk city, Western Siberia. The strain together with other isolates formed a new genetic lineage within TBEV-Sib.

2. Materials and methods

2.1. TBEV-2871 strain isolation and biological properties study

The TBEV-2871 strain was isolated from a female adult *Ixodes pavlovskyi* Pom., 1946 tick collected by flagging in the Akademgorodok district of Novosibirsk city, Western Siberia, Russia (54.78 N, 83.18 E), in 2012. Tick species were morphologically identified according to Filippova (1977). Tick suspension in 0.9% NaCl was injected intracerebrally (i.c., first passage) into ICR mice (6–7 g). In seven days, an acute neuroinfection with convulsions, paresis, paralysis and death of mice was observed. A 10% suspension of the brains of animals demonstrating clinical signs of encephalitis was prepared in 0.9% NaCl for further virological, immunological and genetic studies.

Hemagglutinin was obtained from the brains of infected mice by extraction in borate-salt buffer pH 9.3 (Mel'nikova, 1986). The hemagglutination reaction was carried out using 0.4% goose erythrocyte suspension, as described previously (Clark and Casals, 1958). The species specificity of TBEV hemagglutinin in the hemagglutination inhibition reaction test (HIRT) was confirmed with the preparation of rat polyclonal antibodies against TBEV-FE Sofjin strain (Virion, Russia).

Neurovirulence of TBEV was determined by i.c. (0.03 ml) and s.c. (0.25 ml) administrations of 10-fold serial dilutions of the TBEV-containing brain homogenate to BALB/c mice (8–10 g); animals infected i.c. and s.c. were observed for 21 days. The calculation of the virus titers in lg $LD_{50/ml}$ was performed according to Kärber (1931). The neuroinvasiveness of TBEV was estimated based on the invasiveness index, which was defined as the ratio of virus titers after i.c. and s.c. infection of mice (Pogodina, 1963). The value of invasiveness index between 1 and 2.5 indicates a high invasive activity of the virus, i.e., its ability to overcome the blood-brain barrier, reach central nervous system and propagate in it. An invasiveness index value ≥ 3 indicates a lower invasive activity of the virus strain.

All animal experiments were conducted in compliance with the Animal Welfare Act at the Institute of Systematics and Ecology of Animals SB RAS, Novosibirsk, Russia, according to the guidelines for experiments with laboratory animals (Supplement to the Order of the Russian Ministry of Health, no. 755, of August 12, 1977).

2.2. Total RNA isolation

Total RNA was isolated by a phenol de-proteinization method. Brain samples of laboratory mice infected with the studied TBEV strain were used for preparing 10% suspensions in 600 µl of 0.9% NaCl solution. Then, 60 µl of 10% SDS and 600 µl of phenol saturated with 100 mM sodium acetate (pH 4.5) were added to the suspensions and incubated for 15 min at 65 °C. After incubation, the samples were cooled for 10 min at − 20 °C and then centrifuged for 15 min at 10,000*g*. The upper aqueous phase was transferred, and 1/10 of the total volume of 3 M sodium acetate (pH 4.5) was added, followed by an equal volume of isopropanol, and then incubated for 30 min at − 20 °C. RNAs were precipitated by centrifugation for 10 min at 10,000*g*. After removing the supernatant, the precipitate was washed sequentially with 70% and 96% ethanol. Then, the precipitate containing the total RNA was dried at 37 °C and dissolved in 50 µl of RNase-free water. Isolated RNAs were stored at − 70°C.

2.3. TBEV-2871 strain genome amplification and sequencing

cDNA synthesis and PCR were performed as described previously (Tkachev et al., 2017). For complete genome sequencing, a set of overlapping PCR-fragments was obtained with primers corresponding to the appropriate TBEV genome fragments (Supplement 1). Nucleotide sequences of the PCR products purified with GFX Columns (Amersham Biosciences, USA) were determined in both directions using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). The assembly of PCR-fragments' sequences into full-genome ones was performed with MEGA 7.0 (Kumar et al., 2016).

2.4. Genetic analysis

Homology search was carried out with the BLAST (http://www.ncbi.nlm.nih.gov/BLAST) software using the sequences of different TBEV subtypes available in the Genbank database. Sequence alignment was performed by the ClustalW method with MEGA 7.0 software (Kumar et al., 2016). Identity levels of the sequences were calculated with the Unipro UGENE v. 1.22 software (Okonechnikov et al., 2012). Dendrograms were constructed using the MEGA 7.0 software (Kumar et al., 2016) by a discrete maximum likelihood (ML) (Felsenstein, 1981) method. The choice of the most suitable substitution model for the sequences analysis and dendrogram construction was carried out with jModelTest (Posada, 2008) and MEGA 7.0 (Kumar et al., 2016) software. The significance of the dendrograms was estimated by bootstrap analysis with 1000 replicates. The analysis of possible recombination events in the TBEV-2871 strain sequence was performed with RDP, GENECONV, Chimaera, MaxChi, BootScan, SiScan and 3Seq programs from the RDP software package (Martin et al., 2015).

The rates of nucleotide substitution per site and the divergence times of different genetic lineages were estimated by the Bayesian Markov Chain Monte Carlo (MCMC) method with BEAST 2.4.7 and FigTree 1.4.3 software (Bouckaert et al., 2014; Drummond et al., 2002). A relaxed molecular clock was used since flavivirus evolution generally approximates a molecular clock, but some minor rate differences can occur (Twiddy et al., 2003). Sufficient MCMC chains were run to ensure convergence with an initial 10% of the MCMC chains discarded as burn-in. Statistical uncertainty around the mean estimates was provided by the 95% highest probability density values. To achieve the statistically significant Effective Sample Size (ESS), 40 million iterations were used. The effect of selection on the TBEV E gene (selection test) was evaluated by calculating the average values of non-synonymous/synonymous (dN/dS) frequency ratios using the SLAC (Single Likelihood Ancestor Counting) method (Kosakovsky Pond and Frost, 2005a) available on the Datamonkey server (www.datamonkey.org) (Kosakovsky Pond and Frost, 2005b; Delport et al., 2010).

3. Results

3.1. Biological properties of TBEV-2871 strain

The strain was isolated and maintained by i.c. inoculation into ICR mice. Clinical manifestations of acute encephalitis were observed in mice infected with tick suspension (first passage) and mice brain suspension (second passage) for seven and five days, respectively.

The hemagglutinating antigen was obtained from suspensions of brains of infected mice after first and second passages and then the hemagglutination reaction was carried out with confirmation of the