Ticks and Tick-borne Diseases 12 (2021) 101557

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Short communication

High-throughput sequencing of two European strains of tick-borne encephalitis virus (TBEV), Hochosterwitz and 1993/783

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ARTICLE INFO

Keywords:
TBEV
Flaviviruses
WGS
Phylogeny
Ambiguous nucleotide

ABSTRACT

Tick-borne encephalitis virus (TBEV) is a medically important arbovirus, widespread in Europe and Asia. The virus is primarily transmitted to humans and animals by bites from ticks and, in rare cases, by consumption of unpasteurized dairy products. The aim of this study was to sequence and characterize two TBEV strains with amplicon sequencing by designing overlapping primers. The amplicon sequencing, via Illumina MiSeq, covering nearly the entire TBEV genome, was successful: We retrieved and characterized the complete polyprotein sequence of two TBEV strains, Hochosterwitz and 1993/783 from Austria and Sweden, respectively. In this study the previous phylogenetic analysis of both strains was confirmed to be of the European subtypes of TBEV (TBEV-Eu) by whole genome sequencing. The Hochosterwitz strain clustered with the two strains KrM 93 and KrM 213 from South Korea, and the 1993/783 strain clustered together with the NL/UH strain from the Netherlands. Our study confirms the suitability and rapidness of the high-throughput sequencing method used to produce complete TBEV genomes from TBEV samples of high viral load giving high-molecular-weight cDNA with large overlapping amplicons.

1. Introduction

Tick-borne encephalitis virus (TBEV) is a medically important arthropod-borne virus (arbovirus), which is widespread across large parts of Europe and Asia. TBEV is the causative agent of the disease tick-borne encephalitis (TBE) in humans and animals (Lindquist and Vapalahti, 2008; Suss, 2011). TBEV is mainly transmitted to humans and animals through bites from *Ixodes ricinus* and *Ixodes persulcatus* ticks, and in rare cases through ingestion of unpasteurized dairy products (Balogh et al., 2010; Brockmann et al., 2018; Holzmann et al., 2009; Hudopisk et al., 2013; Kerlik et al., 2018; Paulsen et al., 2019; Ruzek et al., 2019). Some small mammals are proven reservoirs for the TBEV, while migratory birds and large mammals are important for distribution of ticks and the virus (Carpi et al., 2008; Mlera and Bloom, 2018; Nuttall

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https://doi.org/10.1016/j.ttbdis.2020.101557
Received 6 April 2020; Received in revised form 28 August 2020; Accepted 1 September 2020
Available online 14 October 2020
1877-959X/© 2020 Published by Elsevier GmbH.

and Labuda, 2003; Waldenström et al., 2007).

Taxonomically, TBEV belongs to the genus *Flavivirus* within the family *Flaviviridae*. The TBEV genome consist of approximately 11 kb positive-sense single-stranded RNA. The viral RNA encodes one single open reading frame (ORF) of about 3400 amino acids, flanked by 5′ and 3′ non-coding regions (NCRs), three structural proteins; envelope (E), precursor membrane (PrM) and capsid (C), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A-2K-4B complex and NS5) (Heinz and Mandl, 1993; Kaufusi et al., 2014; Plaszczyca et al., 2019; Ruzek et al., 2019; Slavik et al., 1970). Based on phylogenetic grouping and geographical distribution five subtypes of TBEV are known: the European, Siberian, Far Eastern, Baikalian and Himalayan subtypes (Adelshin et al., 2019; Dai et al., 2018; Ecker et al., 1999; Kovalev and Mukhacheva, 2017). These five subtypes are distributed over the Eurasian continent from Europe, Russia, Japan, China and South Korea (Demina et al., 2010; Dobler et al., 2012; Yoshii et al., 2017). The European TBEV subtype (TBEV-Eu) is prevalent across Europe and Asia, from England, France, the Netherlands, to east Sibiria in Russia and South Korea (Dekker et al., 2019; Demina et al., 2010; Dobler et al., 2012; Holding et al., 2019; Ruzek et al., 2019; Velay et al., 2018). Most of the available TBEV-Eu sequences originate from ticks. Conversely, few sequences are available from clinical TBE cases because they often are PCR-negative on the onset of neurological symptoms (Haglund et al., 2003; Saksida et al., 2005).

In this work we sequenced two cultured TBEV strains; one originating from a Swedish patient (1993/783) (Haglund et al., 2003) and one from an Austrian tick collected in 1971 (Hochosterwitz) (Heinz and Kunz, 1981). The aim of this study was to characterize the two TBEV strains. We established a high-throughput amplicon sequencing method with overlapping primers on Illumina MiSeq, based on a protocol developed by Quick et al. (2017).

2. Materials and methods

2.1. Virus strains and virus-cultivation

The Hochosterwitz strain was isolated from an *I. ricinus* tick collected in 1971 near the Hochosterwitz palace in Austria, which is considered a highly endemic TBE area. The strain 1993/783 originated from a patient hospitalized in 1993 at Kalmar Hospital, Sweden, with a moderate form of TBE with symptoms such as fever, malaise, myalgia and headache (Haglund et al., 2003). The Hochosterwitz strain had been passaged two times in mouse brain and then passaged several times in African green monkey kidney Vero E6 cells. Strain 1993/783 had initially been passaged once in suckling baby mice and once in Vero E6 cells. Prior to the experiments, Hochosterwitz were passaged eight times and 1993/783 three times in Vero E6 cell culture. The viral titre for Hochosterwitz was approximately 6.5×10^6 focus forming units according to the protocol by Stiasny et al. (2009) per ml, while the titre for 1993/783 was unknown (Stiasny et al., 2009).

2.2. RNA extraction and reverse transcription (RT) of viral RNA

Viral RNA was extracted from the cultivated virus stocks of the strains using QIAamp® Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations. Immediately after extraction, the viral RNA was reversely transcribed to cDNA using SuperScript III reverse transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with random primers and RNase inhibitor (Applied Biosystems, Foster City, California, USA) according to the manufacturers' protocol.

2.3. Primer design, polymerase chain reaction and gel electrophoresis

To recover the complete coding TBEV genome, we followed the "Primal Scheme" as described in Quick et al. (2017). Briefly, 54 TBEV genomes representing the European TBEV genetic diversity were retrieved from NCBI GenBank and aligned using Muscle 3.8.425 (Edgar, 2004). Primers were designed using the online resource "Primal Scheme" (Quick et al., 2017), with amplicon length set to 2000 nt and overlap to 200 nt (Table 1). PCR was performed following the protocol with the Q5 high fidelity polymerase enzyme described in Quick et al. (2017).

2.4. Library preparation and high-throughput sequencing

The PCR products were cleaned using 1.8x Ampure XP beads (Beckman Coulter Life Sciences, Indianapolis, Indiana) according to the Kapa HyperPlus Kit clean-up protocol (KAPA Biosystems, Roche, Basel, Switzerland). Library preparation and amplification were performed using KAPA HyperPlus (KAPA Biosystems, Roche, Basel, Switzerland) and sequenced using a MiSeq using 2 × 300 v3 (Illumina, San Diego, California), following to the manufacturers' recommendations.

2.5. High-throughput sequence data processing and assembly

Sequencing reads from the separate products representing each strain were concatenated, reads smaller than 50 nt and poor quality reads were removed using Trim Galore v0.4.1 (a wrapper by Felix Krueger at the Babraham Institute using Cutadapt v1.18 (default quality trimming; Q < 30) (Martin, 2011) and FastQC (Simon Andrews also at the Babraham Institute)). Reads from each of the two strains were mapped to a library of TBEV sequences (Table S1) using BowTie2 v2.3.4.3 (with the local alignment option) (Langmead and Salzberg, 2012). Aligned reads were further processed using Samtools v1.9 (Li, 2011) and weeSAM v1.4 (Centre for Virus Research, Glasgow, UK). The highest number of reads from the sequenced Hochosterwitz strain mapped to KrM 93 (HM535611.1), and the 1993/783 strain to NL/UH (MH021184.1), consequently these two were used as references for a second round of reference-based assembly using the same software. Consensus sequences of Hochosterwitz and 1993/783 were called from

Table 1
Primer pairs used to sequence the two tick-borne encephalitis virus strains Hochosterwitz and 1993/783.

Primer name	Position*	Sequences (5' to 3')	Primer pair
JK_1_Forward	35-57	AGC ATT AGC AGC GGT TGG TTT G	Primer pair 1
JK_1_Reverse	1998-1976	GAC TGG GAT CCT ACA GGG CTT T	
JK_2_Forward	1742-1764	CGG AGA CCA GAC TGG AGT GTT A	Primer pair 2
JK_2_ Reverse	3770-3748	AAC ACA GCC TGG AGT AGC ATC A	
JK_3_ Forward	3511-3533	TTG CGG ACA ACG GTG AAT TAC T	Primer pair 3
JK_3_ Reverse	5343-5321	GAA CCT GAC CCG TTT CCC ATT C	
JK_4_ Forward	5077-5099	ATG AGA CCT ACG TCA GCA GCA T	Primer pair 4
JK_4_ Reverse	6918-6896	CAT CTC ATT GGC TGC AAC CAG T	
JK_5_Forward	6671-6693	CTT CGT CGT CCG GAC TTC AAT C	Primer pair 5
JK_5_ Reverse	8660-8638	GGC CAG CTG AGA AGT TTC ACA A	
JK_6_ Forward	8384-8406	ACT TTT GGC TCG GTT TGG AGA C	Primer pair 6
JK_6_ Reverse	10261-10239	CCC AGA TGT TCT TGG CCC ATT C	
JK_7_ Forward	9347-9369	GCA CAA ACA ATT GGC AAC CAC A	Primer pair 7
JK_7_ Reverse	11197-11175	ATT TCT CTC TTC CCT CCT CCC G	

* The positions correspond to the consensus alignment of 54 TBEV genomes, representing European TBEV genetic diversity, retrieved from NCBI GenBank.

the resulting alignment using the built-in consensus caller in Geneious Prime v2020.1, calling bases matching at least 50% of the reads, only reads mapping the region corresponding to the amplified products JK1–JK7 were considered (entire region had >1000 coverage).

2.6. Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment was performed using Muscle 3.8.425 (Edgar, 2004) on genomes described in (Table S1) in addition to one strain from Denmark (Andersen et al., 2019), and 14 sequences from Finland (Smura et al., 2019).

Nucleotide model selection was performed in ModelFinder integrated in IQ-TREE 1.6.11 (Nguyen et al., 2015). The evolutionary history of the complete polyprotein alignment was inferred using the maximum likelihood method with the GTR + F+I + G4 model of nucleotide substitution using IQ-TREE 1.6.11. Branch support expressed as Shimodaira–Hasegawa approximate likelihood-ratio test (SH-aLTR) and ultrafast bootstrap were both calculated by computing 1000 replicates. The Louping ill virus (GenBank accession number: NC_001809.1, strain 369/T2) a closely related flavivirus, was chosen as an outgroup.

3. Results and discussion

The use of amplicon sequencing with overlapping primers has previously been documented as a successful approach to sequence the whole genome of clinical Zika virus samples (Quick et al., 2017). In this study, we designed seven TBEV-Eu primer pairs with fragments of approximately 2000 nt based on the protocol of Quick et al. (2017) and sequenced two TBEV-Eu strains: Hochosterwitz (GenBank accession number MT311861) and 1993/783 (GenBank accession number MT311860). Summary of the sequencing coverage and number of reads is given in Table S2. Since we were sequencing cultivated viruses with high viral load, we designed primers for amplification of longer fragments than Quick et al. (2017). The use of shorter amplicon length, such as 400 nt may be useful for samples with low viral load and/or degraded viral RNA. The amplicon approach allows for multiplexing of samples, and affordable sequencing of the low abundance virus RNA in tick and patient samples.

Both the Hochosterwitz and the 1993/783 sequences contained 10,871 nt, consisting of 76 nt 5’ NCR, 10,245 nt polyprotein, and 551 nt 3’ NCR. The poly(A) tract was mostly deleted (or truncated) but had retained the same pattern of (A)3C(A)6 in both strains. This is identical to the short poly(A) tract retained in the Toro 2003 and Habo 2011 strains. The role of the heterogenic poly(A) tracts in the life cycle of the virus is not clear. However, deep sequencing of Toro 2003 clones after passaging in cell culture or mouse brain revealed mutations in specific genomic regions, indicative of culture driven selection. In addition, mutations within the poly(A) tract are suggested to be an important virulence determinant for TBEV or related to virus cultivation. A longer sequence of the poly(A) tract seemed more common in virus cultivated in mice compared to cell culture (Asghar et al., 2016; Asghar et al., 2014; Mandl et al., 1991). Our sequence result of the NS5 region of 1993/783 showed 100% identity with a previously published sequence of the same region (GenBank accession number KF991109).

Phylogenetic analysis of the complete coding region of Hochosterwitz and 1993/783 confirmed that both strains belong to the TBEV-Eu subtype (Fig. 1). The Hochosterwitz strain grouped together with two endemic South Korean strains, KrM 93 and KrM 213, both belonging to the TBEV-Eu subtype (Yun et al., 2011). This is surprising due to the geographical distance from Europe, and the observation that the neighbouring countries Japan and China mainly harbour the far eastern (TBEV-Fe) subtype (Ko et al., 2010; Yoshii et al., 2017). Migratory birds may have introduced TBEV-Eu strains into South Korea (Carpi et al., 2008; Mlera and Bloom, 2018; Nuttall and Labuda, 2003; Waldenström et al., 2007). Tick populations with TBEV-Eu are found both in Western and Eastern Siberia of Russia (Demina et al., 2010). Considering the relatively short feeding time (five to nine days), it is possible that *Ixodes* ticks may have been acquired by migratory birds at stopovers or transported non-stop over this long-distance (Klaus et al., 2016). There is also a possibility of rodent reservoirs being involved in virus distribution through international sea trade.

The phylogenetic analysis demonstrated that the strain 1993/783 clustered with both the NL/UH strain from the Netherlands, and with the strains from Slovenia (Ljubljana) and Finland (Isosaari) (Fig. 1). The strain 1993/783 and the Slovenia (Ljubljana) strain both originated from human cases. The Swedish patient (1993/783) with a moderate

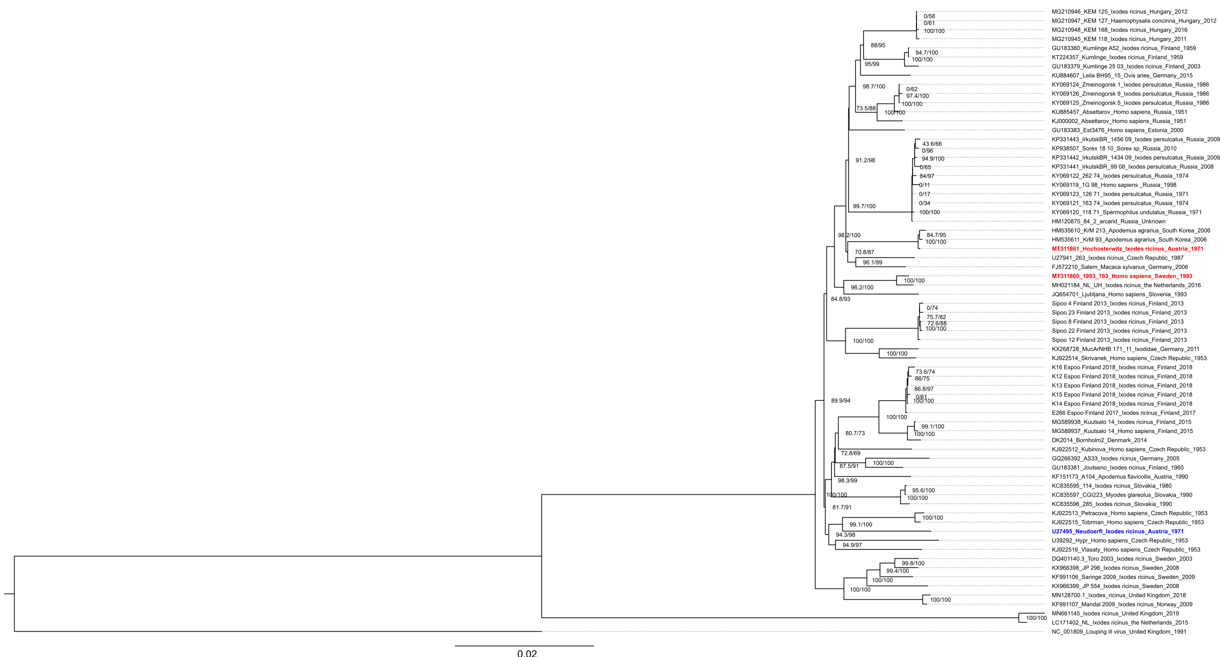

Fig. 1. Maximum likelihood tree showing the TBEV-Eu diversity. Node numbers represent bootstrap values (SH-aLRT support (%) / ultrafast bootstrap support (%)). Scale bar show number of nucleotide changes. Samples in this study show in red, TBEV-Eu reference strain Neudoerfl shown in blue.

TBE disease was presumably infected in the Kalmar municipality in Sweden (Haglund et al., 2003), while the strain from Slovenia (Ljubljana) originated from a severe human TBE case, most likely infected by aerosols while working with TBEV in the laboratory (Avsic-Zupanc et al., 1995).

The various European TBEV strains do generally not display any geographical clustering with regards to their host origin. Strains from the Netherlands, Sweden and Finland belong to different clusters though they all originated from ticks. The 1993/783 strain from the patient did not cluster with the other Nordic strains originating from ticks, like the Finnish (Sipoo and Espoo) or the Swedish strains (Saringe, Torö, JP-296 and JP-554) (Fig. 1). The overall genetic variation of TBEV does not seem to be host dependent since the strains isolated from patients cluster with the tick strains and vice versa (Fig. 1) (Grubaugh et al., 2019). We do not find any geographic clustering in this study, confirming previous studies suggesting a lack of a distinct phylogeographic pattern in TBEV-Eu strains (Heinze et al., 2012).

We identified 52 and eight ambiguous nucleotide positions in the sequence reads of the Hochosterwitz and 1993/783 strains, respectively (Table S3). One of the ambiguous nucleotides, at position 1923, were primer induced. Exchange of amino acids with different biochemical properties affecting polarity or charge due to different folding or protein function, might be critical for the survival, transmission and replication of TBEV. The main ambiguous nucleotides can be explained by either PCR, sequencing induced errors, passage history in laboratory mice and mammalian cell lines or indicate sub-populations or quasispecies (Grubaugh et al., 2019; Romanova et al., 2007). However, the same amplification methods were used for both TBEV strains and, if the minority populations detected here were due to polymerase or sequencing errors, one might expect similar rates in both TBEV strains (Potapov and Ong, 2017). It is difficult to explain the higher number of ambiguous sites in structural genes of Hochosterwitz strain compared to non-structural genes or 1993/783 strain by PCR-errors. We would expect that PCR-errors were more or less evenly distributed in the genome.

In summary, the amplicon sequencing of two TBEV-Eu strains was successful. We retrieved and characterized the complete polyprotein sequence of Hochosterwitz and 1993/783 from Austria and Sweden, respectively.

4. Conclusions

Our study confirms a method for high-throughput sequencing of TBEV samples of high viral load giving high-molecular-weight cDNA with large overlapping amplicons. This offers an improved tool for TBEV sequencing and diagnostics of TBE. The multiplex PCR protocol has advantages as it reduces the cost of reagents and minimises the possibility of laboratory errors. Studying virus populations within naturally infected humans and ticks can lead to breakthrough in our understanding of virus-host interactions and novel approaches for surveillance.

Funding

This study was supported the EU Interreg V-A program Scandtick Innovation project [grant number 20200422]; the Norwegian Ministry of Health and Care Services, Barents region program [grant number B1412 and B1710]; Swedish research council FORMAS [grant number 2015-710].

Declaration of Competing Interest

None

Acknowledgements

Sincere thanks to Sirkka Vene for guidance and cultivation of the TBEV strains, and to Professor Franz X. Heinz, Medical University of Vienna, Austria for kindly providing the Hochosterwitz strain and to Kedar Gautam for contribution to the laboratory work.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101557.

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