Infection, Genetics and Evolution 67 (2019) 150–158

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

A novel genetic lineage of Tula orthohantavirus in Altai voles (*Microtus obscurus*) from Turkey

Ceylan Polat[a], Koray Ergünay[b,*], Sercan Irmak[c], Mert Erdin[a], Annika Brinkmann[d], Ortaç Çetintaş[e], Muhsin Çoğal[e], Mustafa Sözen[e], Ferhat Matur[f], Andreas Nitsche[d], İbrahim Mehmet Ali Öktem[a]

[a] Dokuz Eylul University, Faculty of Medicine, Department of Medical Microbiology, 35340 Izmir, Turkey
[b] Hacettepe University, Faculty of Sciences, Department of Biology, Division of Ecology, Ankara, Turkey
[c] Balıkesir University, Science and Technology Application and Research Center, Balıkesir, Turkey
[d] Robert Koch Institute; Centre for Biological Threats and Special Pathogens 1 (ZBS 1), Berlin, Germany
[e] Bülent Ecevit University, Faculty of Arts and Sciences, Department of Biology, Zonguldak, Turkey
[f] Dokuz Eylul University, Faculty of Science, Department of Biology, Izmir, Turkey

ARTICLE INFO

Keywords:
Hantavirus
Rodentia
Arvicolinae
Turkey

ABSTRACT

Orthohantaviruses (family *Hantaviridae* order *Bunyavirales*) are emerging pathogens with a significant impact on human health. They are transmitted via aerosolized excreta of rodents which also act as reservoir hosts, constituting a unique route for dispersion. Dobrava-Belgrade and Puumala orthohantaviruses have been previously reported from Anatolia, in rodents, case reports and occasional outbreaks. We have collected rodents at several locations during a surveillance study in eastern Anatolia. The specimens were morphologically-identified and various tissues were screened via a generic orthohantavirus reverse transcription polymerase chain reaction assay. DNA barcoding via mitochondrial cytochrome *b* sequencing was performed in rodents with detectable orthohantavirus sequences. High throughput sequencing was performed for viral genome characterization. Fifty rodents were collected and identified morphologically as *Microtus* spp. (96%) and *Apodemus* spp. (4%). Orthohantavirus sequences were detected in lung and spleen or liver tissues of 4 voles (8%), barcoded as *Microtus obscurus*. The virus sequences were identified as Tula orthohantavirus (TULV) and near-complete genomic segments of the prototype viral genome, tentatively named as the Tula orthohantavirus-Turkey (TULV-T), could be characterized. Putative open reading frames for viral nucleocapsid and a nonstructural protein on the S segment, glycoproteins G1 and G2 on the M segment and viral replicase on the L segment were identified on the TULV-T. Several minor sequence variants were further characterized. No evidence of recombination could be detected and pairwise comparisons displayed over 95% amino acid sequence identities to various Eurasian TULV strains. Phylogenetic analyses revealed distinct clustering of all genome segments from previously-characterized TULV strains via various approaches and models. Here, TULV-T constituted a novel lineage, forming an intermediate among Asian and European TULV lineages. This report describes the initial documentation of TULV circulation and its potential reservoir in Anatolia. The extent of virus dispersion, alternate hosts or outcomes of human exposure require elucidation.

1. Introduction

Members of the *Orthohantavirus* genus (family *Hantaviridae* order *Bunyavirales*) are enveloped viruses, possessing a tripartite RNA genome (Whitehouse et al., 2015; Adams et al., 2017). In nature, orthohantavirus hosts are rodents of the *Arvicolinae*, *Murinae*, *Neotominae* and *Sigmodontinae* subfamilies and the viruses are dispersed via virus-containing aerosolized excreta of their rodent hosts, which constitutes a

Abbreviations: bp, base pair; DOBV, Dobrava-Belgrade virus; HCPS, Hantavirus cardiopulmonary syndrome; HFRS, Haemorrhagic fever with renal syndrome; HTS, High throughput sequencing; NSs, Nonstructural protein; nt, Nucleotide; ORF, Open reading frame; PCR, Polymerase chain reaction; PUUV, Puumala virus; RT-PCR, reverse transcription- polymerase chain reaction; TULV, Tula orthohantavirus; TULV-T, Tula orthohantavirus-Turkey

* Corresponding author at: Hacettepe University Faculty of Medicine, Department of Medical Microbiology, Virology Unit, Morphology Building 3rd Floor., 06100, Sihhiye Ankara, Turkey.
E-mail address: ekoray@hacettepe.edu.tr (K. Ergünay).

https://doi.org/10.1016/j.meegid.2018.11.015
Received 24 September 2018; Received in revised form 16 November 2018; Accepted 16 November 2018
Available online 19 November 2018
1567-1348/

unique transmission route among bunyaviruses (Kang et al., 2011; Whitehouse et al., 2015). Currently, over 40 orthohantavirus species have been characterized, where 20 are associated with human disease (Avsic-Zupanc et al., 2015; Briese et al., 2016). Human infections with orthohantaviruses are mostly rodent-borne and may result in severe clinical presentations, namely; haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) (Vaheri et al., 2013; Avsic-Zupanc et al., 2015). Among orthohantaviruses associated with HFRS, Dobrava-Belgrade virus (DOBV) in Europe and Hantaan virus in Asia are responsible for severe forms of HFRS, with 3–12% average mortality rates (Vapalahti et al., 2003; Bi et al., 2008). In Europe, Puumala virus (PUUV) and Saaremaa virus are also associated with milder forms of HFRS (Sironen et al., 2005; Bi et al., 2008). HCPS is mainly caused by New World orthohantaviruses, such as Sin Nombre virus and Andes virus, with higher fatality rates around 40% (Vaheri et al., 2013). With the identification of novel or variant viruses, bat and insectivore infections and regularly documented cases, orthohantaviruses are considered as emerging pathogens with an increasing impact on human health (Guo et al., 2013; Avsic-Zupanc et al., 2015).

Evidence for orthohantavirus circulation in Anatolia have previously been described. DOBV and PUUV have been documented to infect different species of rodents in Anatolia and Thrace (Laakkonen et al., 2006; Oktem et al., 2014; Polat et al., 2018). Moreover, these viruses have been identified as the causative agent in various case reports and occasional outbreaks, mainly from the Black Sea and Thrace regions (Ertek and Buzgan, 2009, Oncul et al., 2011, Sarıgüzel et al., 2012, Gozalan et al., 2013) (Fig. 1). However, detailed information on virus dispersion in various regions of Anatolia is lacking. Moreover, several rodent species, previously documented as reservoirs for various orthohantaviruses were identified in the Anatolian fauna (Yiğit et al., 2006). This study was carried out as an orthohantavirus screening of probable reservoirs via nucleic acid testing in eastern Anatolia.

2. Materials and methods

2.1. Ethics statement

The study was carried out using tissues of field-collected rodents. The design, animal handling and experimental procedures were approved by the Dokuz Eylül University Local Ethical Committee of Animal Experiments (No: 72/2016), and the General Directorate of Nature Conservation and National Parks, Ministry of Forestry and Water Affairs (No: 72784983–488.04-11,467).

2.2. Specimen collection and processing

Live voles were collected at four different locations around Erzurum province (Fig. 1) during October 2016, via Sherman-type traps (H.P. Sherman Traps Inc. Deland, Florida, USA). The animals were sacrified by cervical dislocation, identified morphologically and dissected on site. Tissue specimens were preserved within a commercial RNA stabilization solution (RNAlater, Invitrogen, Carlsbad, CA, USA), transferred in ambient temperature for further storage at −80 °C. Nucleic acids were extracted from the tissues using TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA, USA) and RNA was reverse-transcribed with random hexamers by RevertAid Premium reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Orthohantavirus screening

A previously-reported generic nested orthohantavirus reverse transcription- polymerase chain reaction (RT-PCR) assay was employed for virus screening (Klempa et al., 2006). The assay targets a 390-base pair (bp) sequence in the orthohantavirus L segment, encoding the RNA-dependent RNA polymerase. Each reaction mixture included 2 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of primers, and 2.5 U Taq Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Thermal cycling parameters for the first round were as follows: an initial denaturation at 94 °C for 1 min; 5 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min; 5 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min; 5 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min; 20 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min; and a final extension step of 72 °C for 5 min. Cycling parameters for the second reaction consisted of an initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 1 min, and a final extension step of 72 °C for 5 min. PCR products were visualized by electrophoresis on 1.5% agarose gels. Identically-processed rodent tissues, previously-identified as infected with DOBV were used as positive controls (Polat et al., 2018).

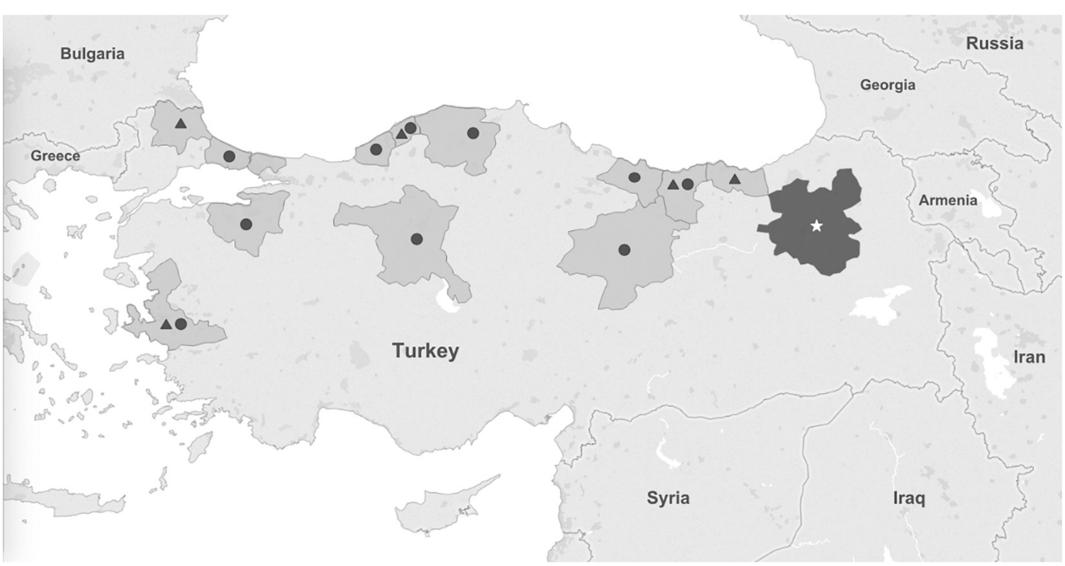

Fig. 1. Illustrative map of Anatolia and eastern Thrace, indicating provinces with previously documented Dobrava-Belgrade and Puumala orthohantavirus activity (Laakkonen et al., 2006; Ertek and Buzgan, 2009; Oncul et al., 2011; Sarıgüzel et al., 2012; Gozalan et al., 2013; Oktem et al., 2014; Polat et al., 2018) and the study region (marked with a star) (black circle: human cases and/or exposure, black triangle: detection in rodents).

Table 1
Sampling locations and specimen distribution in the study.

		Lung		Spleen		Liver		Virus ID	Host ID
Location	Genus	Tested	Positive	Tested	Positive	Tested	Positive		
1 (Latitude:39,826,194 Longitude:41,325,141)	*Microtus*	27	4	4	1	4	1	TULV	*Microtus obscurus*
2 (Latitude:39,8433 Longitude:41,331,943)	*Microtus*	17	0	–	–	–	–	–	–
3 (Latitude:39,891,655 Longitude:41,408,733)	*Microtus*	3	0	–	–	–	–	–	–
	Apodemus	1	0	–	–	–	–	–	–
4 (Latitude:39,84,887 Longitude:41,331,053)	*Microtus*	1	0	–	–	–	–	–	–
	Apodemus	1	0	–	–	–	–	–	–
Total		50	4	4	1	4	1		

2.4. Barcoding in rodents

Rodents with detectable orthohantavirus sequences were subjected to DNA barcoding for precise species identification. For this purpose, the mitochondrial *cytochrome b* gene (1140 bp) was amplified in a single reaction using L14727-SP and H15915-SP primers, as described previously (Jaarola and Searle, 2002). The reaction mix included 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of primers and 1.25 U Taq Polymerase (Thermo Fisher Scientific). Thermal cycling consisted of an initial 5-min denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 1 min, extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. The products were visualized by electrophoresis on 1.5% agarose gels. Previously-collected *Apodemus flavicollis* tissues were employed as positive controls (Polat et al., 2018).

2.5. Sequencing and phylogenetic analyses

Products of the orthohantavirus screening and rodent barcoding assays were characterized by sequencing. Amplicon clean-up was carried out using PureLink PCR Purification Kit (Thermo Fisher Scientific), and the sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and Applied Biosystems 3500xL Dx Genetic Analyzer (Thermo Fisher Scientific). Obtained sequences were handled using Geneious software v11.1.5 (Biomatters Ltd., Auckland, New Zealand).

Nucleotide sequence similarity searches in the public databases were assessed by the Basic Local Alignment Search Tool, implemented in the National Center for Biotechnology Information website (www.ncbi. nlm.nih.gov/blast/), using BLASTn, and BLASTn optimized for highly similar sequences (MEGABLAST) and BLASTp, algorithms (Altschul et al., 1990). Nucleotide and amino acid sequence alignments and pairwise comparisons were generated via the CLUSTAL W program (Thompson et al., 1994), implemented in the Geneious software (Biomatters Ltd). Nucleotide similarity plots were generated by SimPlot version 3.5.1 (Lole et al., 1999). Protein domain and motif screening were performed using the web CD-search tool (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) and MOTIF Search (http://www.genome.jp/tools/motif/) in the PFAM database (Bateman et al., 2002; Marchler-Bauer et al., 2015). Screening for recombination among orthohantaviruses was carried out using the algorithms implemented in the RDP4 software (Martin et al., 2010), in the default settings. Phylogenetic analyses were conducted using MEGA6 (Tamura et al., 2013). Evolutionary history was inferred via the maximum-likelihood method based on the model, estimated as the optimal substitution model individually for each alignment according to the Bayesian information criterion. Bayesian phylogenetic inference using the Markov chain Monte Carlo methods were carried out via MrBayes software (Ronquist et al., 2012), using the GTR + I + G model, as determined by jModelTest (Posada, 2008), for 10^7 generations with sample frequency of 10^2. The trees were visualized by FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/)

2.6. High throughput sequencing and data analysis

The cDNA specimens used for orthohantavirus screening were processed via NexteraXT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA), for fragmentation, adaptor ligation and amplification. The sequencing was performed in one lane of the Illumina HiSeq 1500 instrument (Illumina Inc.) in the paired end mode. Trimmed reads were aligned to the RefSeq viral nucleotide and protein genome databases using MALT (MEGAN alignment tool, v0.3.8) (Huson et al., 2016) and DIAMOND (v0.7.1) (Buchfink et al., 2015) tools. Further sequence handling was carried out using the Geneious software (Biomatters Ltd).

3. Results

3.1. Rodents and screening findings

A total of 50 rodents were collected and evaluated. They were identified morphologically as *Microtus* spp. (48/50, 96%) and *Apodemus* spp. (2/50, 4%). Lung tissues from all rodents were screened by the generic orthohantavirus RT-PCR, that yielded products of the expected size in 4 tissues (4/50, 8%). Spleen and liver tissues were available in voles with reactive lung tissues. These were further tested, and produced amplicons in one spleen and one liver tissue (Table 1). The voles with detectable TULV sequences were identified as *Microtus obscurus,* based on the BLASTn identities (GenBank accessions: MK107998, MK107999 and MK108000).

Sequencing of the products provided 352–381 nt partial L segments with 90.6–100% identities. The sequences characterized in various tissues of individual rodents were identical. They demonstrated the highest BLASTn identities to Tula orthohantavirus (TULV) strains, previously-detected in *Microtus arvalis* voles. In the maximum likelihood analysis, the sequences formed a distinct cluster with a TULV strain from *Microtus arvalis* voles in Russia, sharing a common ancestor with viruses from *Microtus* and *Arvicola* voles from Germany (Fig. 2).

3.2. Genome characterization of TULV

The cDNA from a positive lung specimen (no. 8101) was processed for high throughput sequencing (HTS), that produced 3,660,396 reads with 30.62–30.79 mean quality PF scores. Sequences belonging in three viral genomic segments could be characterized; with 1154, 1389 and 1405 reads for the S, M and L segments, respectively. The virus was tentatively named as Tula virus-Turkey (TULV-T), with the isolate number 8101, after the original specimen code.

The S segment sequence (GenBank accession: MH649270) was assembled as 1817 nt, with the 1293 nt putative open reading frame (ORF), encoding for the 430 amino acid viral protein, flanked by 41 and 483 nt non-coding regions in the 5′ and 3′ ends. The functional analysis of the putative protein revealed complete coverage of the

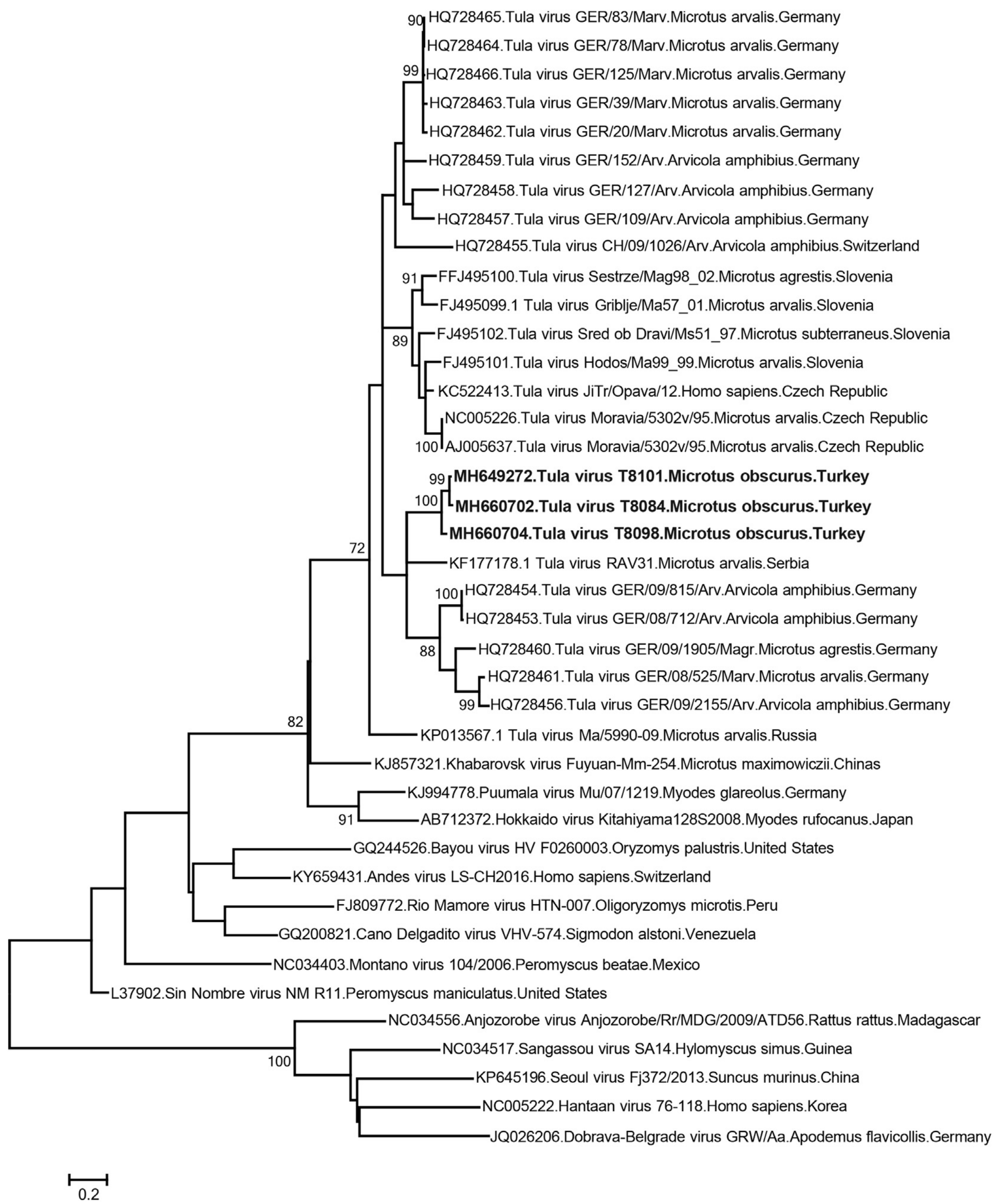

Fig. 2. Phylogenetic analysis of the TULV-T partial L segment sequences (267 nucleotides). The tree is constructed using Maximum likelihood method with the General Time Reversible (GTR) model, Gamma distributed with Invariant sites (G + I) for 1000 replications. Viruses are indicated by GenBank accession number, strain name, host and country of origin. Bootstrap values lower than 70 are hidden.

orthohantavirus nucleocapsid (pfam00846, superfamily cl02985). Pairwise comparisons of the coding region revealed highest identities with TULV strains from various origins, with 83.1–85.5% nt and 95–96.9% amino acid identities (Supplementary file 1). HTS also revealed a distinct minor variant, occurring in 5′ non-coding end (Table 2). The specific amino acid motif (T274Q276T281), previously described in TULV nucleocapsid sequences from Kazakhstan (Plyusnina et al., 2008), was not observed, which appeared as A274R276A281 in TULV-T. In addition to the viral nucleocapsid, TULV-T was observed to encode a putative nonstructural protein (NSs), as noted for vole-derived TULV strains. The TULV-T NSs was located in the 82–354 nt of the S segment and comprised 90 amino acids with 72.2–81.1% identity to various TULV strains (Supplementary file 2). In the phylogenetic analysis based on the complete nucleocapsid protein, TULV-T formed a distinct lineage among viruses from Europe and Russia (Fig. 3). A similar tree topology was observed when partial S nucleocapsid sequences were analysed (Fig. 4). The similarity plot revealed a comparable distribution of diversity among viruses of different origins (Fig. 5). A relatively increased sequence variability was observed within a 200 nt region located in 650–850. positions of the S segment, without coinciding with the NSs coding region (Fig. 5).

A 3458-nt sequence (GenBank accession: MH649271) constituted the viral M genomic segment. It covered the complete ORF of 3426 nt, with a 32 nt stretch at the 3′ end. No information on the 5′-end could be obtained. The deduced putative polyprotein included conserved domains of orthohantavirus glycoprotein G1 (amino acids: 20–547, pfam01567, superfamily cl03268), immunoreceptor tyrosine-based activation motif (ITAM) (amino acids: 616–639, pfam10538, superfamily cl11170) and orthohantavirus glycoprotein G2 (amino acids: 655–1138, pfam01561, superfamily cl03264). Pairwise comparisons

Table 2
Minor sequence variants identified in the Tula virus-Turkey genome. Variations with a detection frequency above 5% are provided. The numbers in parantheses indicate amino acid substitution positions on the deduced viral proteins.

	Position	Predominant Nucleotide	Minor variant	Frequency	Outcome
S segment (1817 nt)	30	A	G	0.2105	unknown (non-coding)
M segment (3426 nt)	59	G	A	0.0556	S-F (1123)
	1068	C	A	0.0541	V-F (787)
	1260	G	A	0.0513	H-Y (723)
	1572	G	A	0.0566	L-F (619)
	2004	G	A	0.05	Silent
	3151	A	G	0.0513	Silent
L segment (6408 nt)	1925	T	A	0.0556	Silent
	3206	A	G	0.0536	Silent
	4376	A	G	0.065	Silent
	5387	A	C	0.0541	K-N (1790)

revealed 81.8–81.9% nt and 97.1–97.2% amino acid identities with TULV strains Moravia/5302v/95 and Moravia/5286 Ma/94. Six minor variants were observed on the M segment, four of which produced amino acid substitutions (Table 2). In the maximum likelihood tree, the complete M glycoprotein precursor grouped with TULV strains with very high bootstrap values, sharing a common ancestor with strains from Czech Republic (Supplementary File 3).

Finally, the L segment of the TULV genome was obtained as near complete ORF (6408 nt; GenBank accession: MH649272), encoding for the 2135 of the 2153-amino acid viral polymerase. Conserved domains of RNA dependent RNA polymerase (amino acids: 188–221, pfam12426, superfamily cl13813) with the conserved MFNLKF motif, cap-snatching RNA endonuclease (amino acids: 84–154, TIGR04202, superfamily cl27920) and bunyavirus RNA dependent RNA polymerase (amino acids: 552–1275, pfam04196, superfamily cl20265) were identified on the putative protein. The sequence revealed 79.5% nt and 96.5% amino acid identities with the available TULV strain (Moravia/5302v/95) in pairwise comparisons. Four minor sequence variants were noted, one of which resulted in an amino acid substitution (Table 2). In the maximum likelihood analysis, a distinct positioning of the putative viral polymerase sequence was observed (Supplementary File 4). No evidence of putative recombination could be detected in any of the segments, via several algorithms. Similar tree configurations were observed for all genome segments in the Bayesian phylogenetic inferences and amino acid based analyses (data not provided).

4. Discussion

During an orthohantavirus screening in field-collected rodent tissues via PCR, we have detected partial TULV L segment sequences in various tissues of Microtus voles (Table 1). HTS of the reactive tissues provided near-complete genome of the infecting strain. This report constitutes the first documentation of TULV circulation in Anatolia.

Initially identified in the Tula region of Central Russia, TULV has been shown to be a widely-dispersed orthohantavirus in Eurasia and has been reported from several countries including Austria, Belgium, Czech Republic, Croatia, France, Germany, Kazakhstan, Poland, Serbia, Slovakia and Switzerland (Plyusnin et al., 1995; Bowen et al., 1997; Heyman et al., 2002; Klempa et al., 2003; Song et al., 2004; Plyusnina et al., 2007; Plyusnina et al., 2008; Schmidt et al., 2016; Nikolic et al., 2014). In accordance with other orthohantaviruses, TULV genome is partitioned into S, M and L segments that constitute 1831, 3694 and 6541 nt, respectively, in the prototype strain Moravia (Vapalahti et al., 1996; Kukkonen et al., 1998). We could obtain complete coding sequences of the S and M segments, as well as the majority of the L segment (99.2% coverage on the deduced protein), via direct HTS. The non-coding terminal regions of the viruses within *Bunyavirales* order are family-specific, forming panhandle-like structures due to base complementary, which facilitates viral genome replication (Whitehouse et al., 2015). Despite successful characterization of the significant portion of the non-coding region of the TULV-T S segment, orthohantavirus specific sequences were not observed, indicating incomplete terminal ends. We have made no further attempt to complete the sequence gaps in TULV-T genomic segments. Nevertheless, the available sequence data enabled comprehensive functional and phylogenetic assessment. ORFs identified on the TULV-T S, M and L segments provided putative domains of nucleocapsid, envelope glycoproteins G1 and G2, as well as viral replicase (Whitehouse et al., 2015). All major putative viral proteins display over 95% identities to available TULV sequences, confirming the identity of the strain as TULV, as previously suggested criteria by the International Committee on Taxonomy of Viruses require at least 7% difference in nucleocapsid and glycoprotein sequences for species delineation (King et al., 2011). The TULV-T further codes for an accessory NSs, previously reported to inhibit activation of the interferon-beta promoter and enhance virus survival in interferon-competent cells (Jääskeläinen et al., 2007; Jääskeläinen et al., 2008). Phylogenetic analyses of the TULV-T revealed that coding regions of all genome segments remained distinct from previously-characterized TULV strains and orthohantaviruses via various approaches and models. Interestingly, in the complete S segment analyses, TULV-T emerged as a separate TULV clade, distantly related to virus strains from Russia, while sharing common ancestors with particular strains from Kazakhstan, Czech Republic, France, Serbia, Slovakia, Poland and Germany (Fig. 2). Comparable findings were also noted in the analysis of the partial L segment sequences obtained from the screening PCR as well as partial S segment analyses, where all sequences characterized in Turkey formed distinct clades (Figs. 3 and 4). This is further supported by M and L coding analyses, albeit with an underrepresentation of geographically-segregated strains. Nevertheless, these findings indicate that TULV-T represents a separate, novel lineage among viruses from Europe and Asia. Previous reports have already provided preliminary evidence of genetic clustering among TULVs, which appears as geographically-associated trait, independent of the host rodent species (Plyusnina et al., 2008; Schmidt et al., 2016). Analysis of the partial S segment sequences have identified the circulation of at least six lineages, represented by viruses of Russian origin (Tula-Central Russia, Omsk-West Siberia and Kazakhstan), a lineage including strains from Germany and Poland, another with strains from East Slovakia and Serbia, and finally by sequences from Croatia and Central Europe (Germany, Switzerland, West Slovakia and Czech Republic) (Plyusnina et al., 2008). We also observed a comparable clustering of strains and distinct loca via analysis of partial S segment sequences (Fig. 4). Our analyses based on complete or partial nucleocapsid coding sequences confirm these findings on a larger scale, as TULVs from Asia, eastern and western Europe form well-supported lineages and TULV-T constitutes an intermediate between major virus lineages. As more complete or near-complete L and M segment sequences become available, the actual state and implications of TULV genetic diversity will be better elucidated. The recent description of the Adler orthohantavirus, a variant of TULV in *Microtus majori* voles in European Russia demonstrates the co-circulation of genetically-related viruses in various rodent species in identical habitats (Tkachenko et al., 2015).

We have detected TULV-T sequences in voles of the *Microtus* spp., that were further characterized via *cytochrome b* barcoding as *M. obscurus*, the Altai vole. The common vole (*M. arvalis*) is generally considered as the primary TULV reservoir (Plyusnin et al., 1995; Plyusnina et al., 2008). However, the virus has so far been detected in several *Arvicolinae* species including *Microtus subterraneus* (*Pitymys subterraneus*), *Microtus levis* (formerly rossiaemeridionalis), *Microtus agrestis*, *Microtus gregalis*, *Arvicola amphibius* and *Lagurus lagurus* (Song et al., 2002; Schmidt-Chanasit et al., 2010; Schlegel et al., 2012). The *Microtus* genus represents the best phylogenetically supported lineage

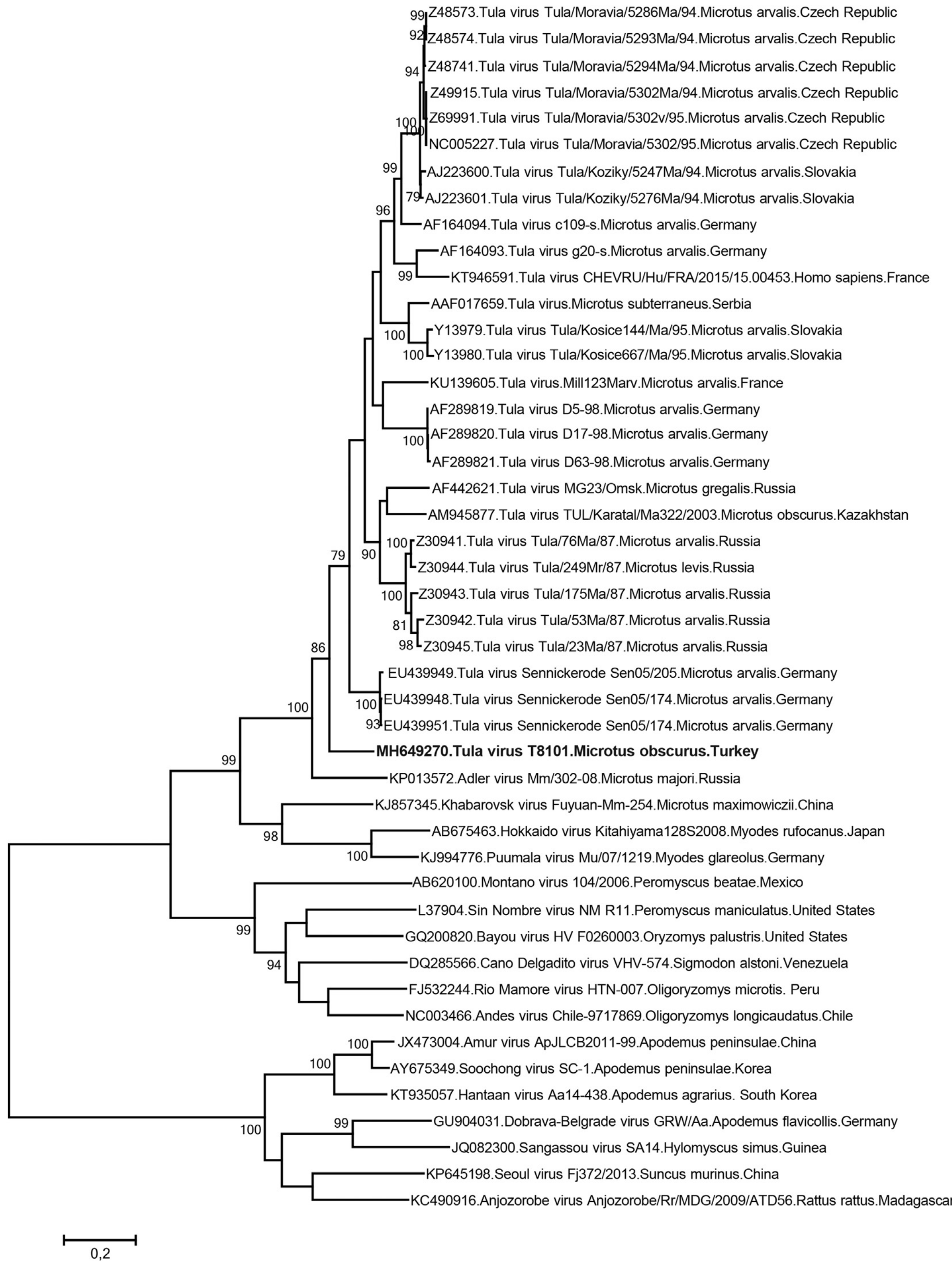

Fig. 3. Phylogenetic analysis of the TULV-T near complete S segment sequences (1659 nucleotides). The tree is constructed using Maximum Likelihood method with the General Time Reversible (GTR) model, Gamma distributed with Invariant sites (G + I) for 1000 replications. Viruses are indicated by GenBank accession number, strain name, host and country of origin. Bootstrap values lower than 70 are hidden.

via *cytochrome b* barcoding and displays close relationships among members of the arvalis species group, which is further concordant with the morphologically cryptic species (Jaarola et al., 2002). In central Europe, TULV is associated with the common vole as the preferential host on a large geographical scale, albeit with regional discrepancies in various contact areas, due to spillover infections in indigenous field and water voles (Schmidt-Chanasit et al., 2010; Schmidt et al., 2016). *M. obscurus* and *M. arvalis* are regarded as sibling species, distributed mainly in eastern and western Eurasia, respectively (Tougard et al., 2013). *M. obscurus* was previously reported as the carrier of TULV in Kazakhstan (Plyusnina et al., 2008). Despite infection in identical hosts, viral sequences detected in Turkey and Asia from *M. obscurus* are phylogenetically-divergent (Figs. 3 and 4), indicating virus adaptation to local reservoirs. It remains to be determined whether TULV infections also occur in vole species other than *M. obscurus* in Anatolia.

Current information on the pathogenicity and health impact of TULV for humans is limited. Despite being considered as a non-pathogenic virus previously, several reports have revealed human exposure and symptomatic infections due to TULV. Virus specific antibodies have been demonstrated in healthy blood donors in the Czech Republic, forestry workers as well as an HFRS patient from Germany (Vapalahti et al., 1996; Klempa et al., 2003; Mertens et al., 2011). In addition,

Fig. 4. Phylogenetic analysis of the TULV-T partial S segment sequences (312 nucleotides). The tree is constructed using Maximum Likelihood method with the General Time Reversible (GTR) model, Gamma distributed with Invariant sites (G + I) for 1000 replications. Viruses are indicated by GenBank accession number, strain name, host and country of origin. Bootstrap values lower than 70 are hidden.

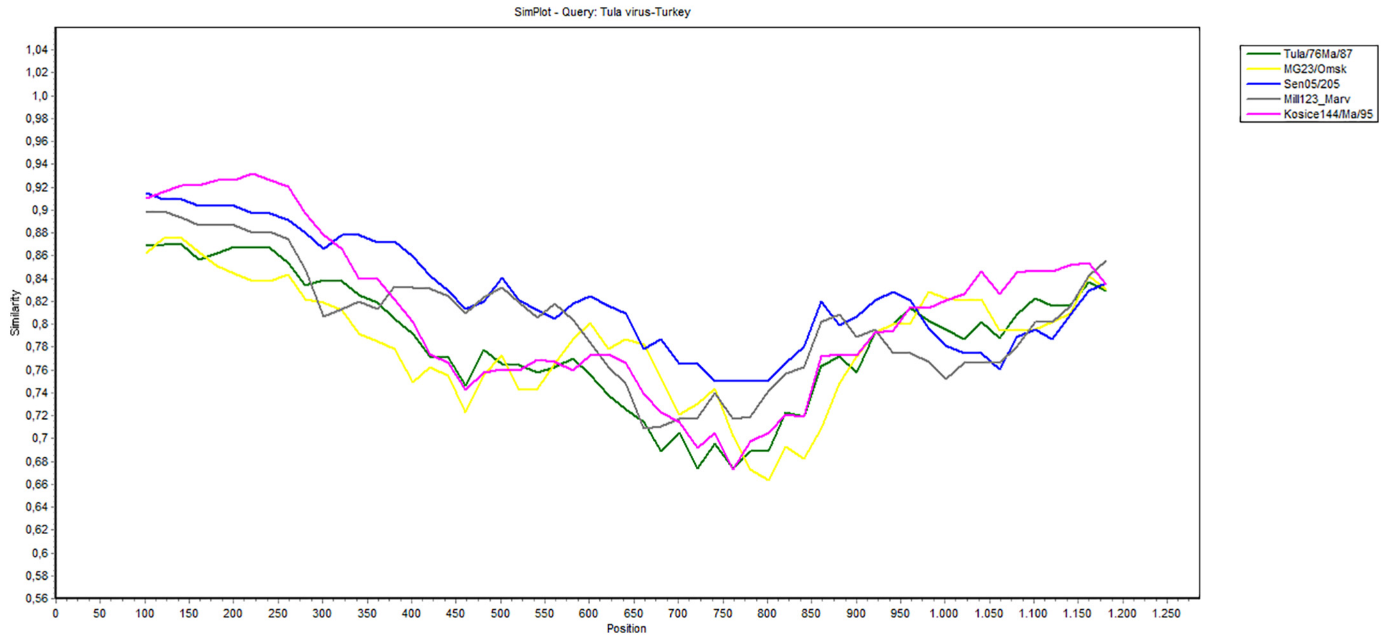

Fig. 5. Plots of similarity of the complete coding S segment alignment (1286 bp) of Tula virus - Turkey (MH649270) (GapStrip: On, Reps: 1000, F84 "Maximum Likelihood", T/t: 2.0). The curves indicate comparisons between the target and reference genomes (GenBank accessions Tula/76 Ma/87: Z30941, MG23/Omsk: AF442621, Sen05/205: EU439951, Mill123_Marv: KU139605, Kosice144/Ma/95: Y13979). Each point plotted is the percent identity within a sliding window 200 bp wide centered on the position plotted, with a step size between points of 20 bp.

TULV RNA was detected in circulation of an immunocompromised person presenting with pulmonary-renal syndrome from Czech Republic and in an HFRS patient with no significant medical history from France (Zelena et al., 2013; Reynes et al., 2015). These reports clearly indicate that symptomatic human infections occur in regions with virus circulation. A detailed description of TULV dispersion in Anatolia, alternate rodent hosts and outcomes of human exposure require further investigations.

5. Conclusion

We hereby describe the initial documentation of TULV circulation and its potential reservoir in Anatolia. The near-complete genome of the prototype strain TULV-T was characterized. The prototype genome constituted a novel lineage, forming an intermediate among Asian and European lineages.

Acknowledgements

The authors are grateful to N. Emin Güven for support in graphic production. Preliminary findings of this study have been presented at the XXXVIII. Turkish Microbiology Congress, to be held from November 4 to 8, 2018, in Antalya, Turkey.

Funding

This study did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.11.015.

References

Adams, M.J., Lefkowitz, E.J., King, A.M.Q., Harrach, B., Harrison, R.L., Knowles, N.J., Kropinski, A.M., Krupovic, M., Kuhn, J.H., Mushegian, A.R., Nibert, M., Sabanadzovic, S., Sanfaçon, H., Siddell, S.G., Simmonds, P., Varsani, A., Zerbini, F.M., Gorbalenya, A.E., Davison, A.J., 2017. Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses. Arch. Virol. 162, 2505–2538.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Avsic-Zupanc, T., Saksida, A., Korva, M., 2015. Hantavirus infections. Clin. Microbiol. Infect (pii: S1198-743X(15)00536–4). https://doi.org/10.1111/1469-0691.12291.

Bateman, A., Birney, E., Cerruti, L., Durbin, R., Etwiller, L., Eddy, S.R., Griffiths-Jones, S., Howe, K.L., Marshall, M., Sonnhammer, E.L., 2002. The Pfam protein families database. Nucleic Acids Res. 30, 276–280.

Bi, Z., Formenty, P.B., Roth, C.E., 2008. Hantavirus infection: a review and global update. J. Infect. Dev. Ctries. 2, 3–23. https://doi.org/10.3855/jidc.317.

Bowen, M.D., Gelbmann, W., Ksiazek, T.G., Nichol, S.T., Nowotny, N., 1997. Puumala virus and two genetic variants of Tula virus are present in Austrian rodents. J. Med. Virol. 53, 174–181(https://doi.org/10.1002/(SICI)1096-9071(199710)53:2 < 174::AID-JMV11 > 3.0.CO;2-J).

Briese, T., Alkhovsky, S., Beer, M., Calisher, C.H., Charrel, R., Ebihara, H., Jain, R., Kuhn, J., Lambert, A., Maes, P., Nunes, M., Plyusnin, A., Schmaljohn, C., Tesh, R., Yeh, S.D., 2016. ICTV taxonomic proposal 2016.023a-cM.A.v2.Hantavirus_sprev. In the genus Hantavirus, create 24 species and abolish 7 species; change the genus name to Orthohantavirus and rename its constituent species similarly. (http://www.ictv.global/proposals16/2016.023a-cM.A.v2.Hantavirus_sprev.pdf. accessed 15 December 2017).

Buchfink, B., Xie, C., Huson, D.H., 2015. Fast and sensitive protein alignment using DIAMOND. Nat. Methods 12, 59–60. https://doi.org/10.1038/nmeth.3176.

Ertek, M., Buzgan, T., 2009. An outbreak caused by hantavirus in the Black Sea region of Turkey, January-May 2009. Euro. Surveill. 14 (20). https://doi.org/10.2807/ese.14.20.19214-en. (pii: 19214).

Gozalan, A., Kalaycioglu, H., Uyar, Y., Sevindi, D.F., Turkyilmaz, B., Çakir, V., Cindemir, C., Unal, B., Yağçi-Çağlayik, D., Korukluoglu, G., Ertek, M., Heyman, P., Lundkvist, Å., 2013. Human puumala and dobrava hantavirus infections in the Black Sea region of Turkey: a cross-sectional study. Vector. Borne. Zoonotic. Dis. 13, 111–118. https://doi.org/10.1089/vbz.2011.0939.

Guo, W.P., Lin, X.D., Wang, W., Tian, J.H., Cong, M.L., Zhang, H.L., Wang, M.R., Zhou, R.H., Wang, J.B., Li, M.H., Xu, J., Holmes, E.C., Zhang, Y.Z., 2013. Phylogeny and origins of hantaviruses harbored by bats, insectivores, and rodents. PLoS Pathog. 9 (2), e1003159. https://doi.org/10.1371/journal.ppat.1003159.

Heyman, P., Klingström, J., De Jaghere, F., Leclercq, G., Rozenfeld, F., Escutenaire, S., Vandenvelde, C., Zizi, M., Plyusnin, A., Lundkvist, A., 2002. Tula hantavirus in Belgium. Epidemiol. Infect. 128, 251–256. https://doi.org/10.1017/S0950268801006641.

Huson, D.H., Beier, S., Flade, I., Gorska, A., El-Hadidi, M., Mitra, S., Ruscheweyh, H.J., Tappu, R., 2016. MEGAN community edition - interactive exploration and analysis of large-scale microbiome sequencing data. PLoS Comput. Biol. 12, e1004957. https://doi.org/10.1371/journal.pcbi.1004957.

Jaarola, M., Searle, J.B., 2002. Phylogeography of field voles (*Microtus agrestis*) in Eurasia inferred from mitochondrial DNA sequences. Mol. Ecol. 11 (12), 2613–2621. https://

doi.org/10.1046/j.1365-294X.2002.01639.x.
Jääskeläinen, K.M., Kaukinen, P., Minskaya, E.S., Plyusnina, A., Vapalahti, O., Elliott, R.M., Weber, F., Vaheri, A., Plyusnin, A., 2007. Tula and Puumala hantavirus NSs ORFs are functional and the products inhibit activation of the interferon-beta promoter. J. Med. Virol. 79 (10), 1527–1536. https://doi.org/10.1002/jmv.20948.
Jääskeläinen, K.M., Plyusnina, A., Lundkvist, A., Vaheri, A., Plyusnin, A., 2008. Tula hantavirus isolate with the full-length ORF for nonstructural protein NSs survives for more consequent passages in interferon-competent cells than the isolate having truncated NSs ORF. Virol. J. 5, 3. https://doi.org/10.1186/1743-422X-5-3.
Kang, H.J., Bennett, S.N., Hope, A.G., Cook, J.A., Yanagihara, R., 2011. Shared ancestry between a newfound mole-borne hantavirus and hantaviruses harbored by cricetid rodents. J. Virol. 85, 7496–7503. https://doi.org/10.1128/JVI.02450-10.
King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J., 2011. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego.
Klempa, B., Meisel, H., Rath, S., Bartel, J., Ulrich, R.G., Krüger, D.H., 2003. Occurrence of renal and pulmonary syndrome in a region of Northeast Germany where Tula hantavirus circulates. J. Clin. Microbiol. 41, 4894–4897. https://doi.org/10.1128/JCM.41.10.4894-4897.2003.
Klempa, B., Fichet-Calvet, E., Lecompte, E., Auste, B., Aniskin, V., Meisel, H., Denys, C., Koivogui, L., Meulen, J., Krüger, D., 2006. Hantavirus in African wood mouse, Guinea. Emerg. Infect. Dis. 12, 838–840. https://doi.org/10.3201/eid1205.051487.
Kukkonen, S.K., Vaheri, A., Plyusnin, A., 1998. Completion of the Tula hantavirus genome sequence: properties of the L segment and heterogeneity found in the 3′ termini of S and L genome RNAs. J. Gen. Virol. 79, 2615–2622. https://doi.org/10.1099/0022-1317-79-11-2615.
Laakkonen, J., Kallio-Kokko, H., Oktem, M.A., Blasdell, K., Plyusnina, A., Niemimaa, J., Karataş, A., Plyusnin, A., Vaheri, A., Henttonen, H., 2006. Serological survey for viral pathogens in Turkish rodents. J. Wildl. Dis. 42 (3), 672–676. https://doi.org/10.7589/0090-3558-42.3.672.
Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W., Ray, S.C., 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J. Virol. 73 (1), 152–160.
Marchler-Bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M., Hurwitz, D.I., Lanczycki, C.J., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Bryant, S.H., 2015. CDD: NCBI's conserved domain database. Nucleic Acids Res. 43, D222–D226. https://doi.org/10.1093/nar/gku1221.
Martin, D.P., Lemey, P., Lott, M., Moulton, V., Posada, D., Lefeuvre, P., 2010. RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics 26, 2462–2463. https://doi.org/10.1093/bioinformatics/btq467.
Mertens, M., Hofmann, J., Petraityte-Burneikiene, R., Ziller, M., Sasnauskas, K., Friedrich, R., Niederstrasser, O., Kruger, D.H., Groschup, M.H., Petri, E., Werdermann, S., Ulrich, R.G., 2011. Seroprevalence study in forestry workers of a non-endemic region in eastern Germany reveals infections by Tula and Dobrava-Belgrade hantaviruses. Med. Microbiol. Immunol. 200, 263–268. https://doi.org/10.1007/s00430-011-0203-4.
Nikolic, V., Stajkovic, N., Stamenkovic, G., Cekanac, R., Marusic, P., Siljic, M., Gligic, A., Stanojevic, M., 2014. Evidence of recombination in Tula virus strains from Serbia. Infect. Genet. Evol. 21, 472–478. https://doi.org/10.1016/j.meegid.2013.08.020.
Oktem, I.M., Uyar, Y., Dincer, E., Gozalan, A., Schlegel, M., Babur, C., Celebi, B., Sozen, M., Karatas, A., Ozkazanc, N.K., Matur, F., Korukluoglu, G., Ulrich, R.G., Ertek, M., Ozkul, A., 2014. Dobrava-Belgrade virus in *Apodemus flavicollis* and *A. uralensis* mice, Turkey. Emerg. Infect. Dis. 20, 121–125. https://doi.org/10.3201/eid2001.121024.
Oncul, O., Atalay, Y., Onem, Y., Turhan, V., Acar, A., Uyar, Y., Caglayik, D.Y., Ozkan, S., Gorenek, L., 2011. Hantavirus infection in Istanbul, Turkey. Emerg. Infect. Dis. 17 (2), 303–304. https://doi.org/10.3201/eid1702.100663.
Plyusnin, A., Cheng, Y., Vapalahti, O., Pejcoch, M., Unar, J., Jelinkova, Z., Lehväslaiho, H., Lundkvist, A., Vaheri, A., 1995. Genetic variation in Tula hantaviruses: sequence analysis of the S and M segments of strains from Central Europe. Virus Res. 39, 237–250. https://doi.org/10.1016/0168-1702(95)00086-0.
Plyusnina, A., Deter, J., Charbonnel, N., Cosson, J.-F., Plyusnin, A., 2007. Puumala and Tula hantaviruses in France. Virus Res. 129, 58–63. https://doi.org/10.1016/j.virusres.2007.04.023.
Plyusnina, A., Laakkonen, J., Niemimaa, J., Henttonen, H., Plyusnin, A., 2008. New Genetic Lineage of Tula Hantavirus in *Microtus arvalis obscurus* in Eastern Kazakhstan. Open. Virol. J. 2, 32–36. https://doi.org/10.2174/1874357900802010032.
Polat, C., Sironen, T., Plyusnina, A., Karatas, A., Sozen, M., Matur, F., Vapalahti, O., Oktem, I.M.A., Plyusnin, A., 2018. Dobrava hantavirus variants found in *Apodemus flavicollis* mice in Kırklareli Province, Turkey. J. Med. Virol. 90 (5), 810–818. https://doi.org/10.1002/jmv.25036.
Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25 (7), 1253–1256. https://doi.org/10.1093/molbev/msn083.
Reynes, J.M., Carli, D., Boukezia, N., Debruyne, M., Herti, S., 2015. Tula hantavirus infection in a hospitalised patient, France, June 2015. Euro. Surveill. 20 (50), 30095. https://doi.org/10.2807/1560-7917.
Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61 (3), 539–542. https://doi.org/10.1093/sysbio/sys029.
Sarıgüzel, N., Hofmann, J., Canpolat, A.T., Türk, A., Ettinger, J., Atmaca, D., Akyar, I., Yücel, S., Arıkan, E., Uyar, Y., Çağlayık, D.Y., Kocagöz, A.S., Kaya, A., Kruger, D.H., 2012. Dobrava hantavirus infection complicated by panhypopituitarism, Istanbul, Turkey, 2010. Emerg. Infect. Dis. 18 (7), 1180–1183. https://doi.org/10.3201/eid1807.111746.
Schlegel, M., Kindler, E., Essbauer, S.S., Wolf, R., Thiel, J., Groschup, M.H., Heckel, G., Oehme, R.M., Ulrich, R.G., 2012. Tula virus infections in the Eurasian water vole in Central Europe. Vector. Borne. Zoonotic. Dis. 12, 503–513. https://doi.org/10.1089/vbz.2011.0784.
Schmidt, S., Saxenhofer, M., Drewes, S., Schlegel, M., Wanka, K.M., Frank, R., Klimpel, S., von Blanckenhagen, F., Maaz, D., Herden, C., Freise, J., Wolf, R., Stubbe, M., Borkenhagen, P., Ansorge, H., Eccard, J.A., Lang, J., Jourdain, E., Jacob, J., Marianneau, P., Heckel, G., Ulrich, R.G., 2016. High genetic structuring of Tula hantavirus. Arch. Virol. 161, 1135–1149. https://doi.org/10.1007/s00705-016-2762-6.
Schmidt-Chanasit, J., Essbauer, S., Petraityte, R., Yoshimatsu, K., Tackmann, K., Conraths, F.J., Sasnauskas, K., Arikawa, J., Thomas, A., Pfeffer, M., Scharninghausen, J.J., Splettstoesser, W., Wenk, M., Heckel, G., Ulrich, R.G., 2010. Extensive host sharing of central European Tula virus. J. Virol. 84, 459–474. https://doi.org/10.1128/JVI.01226-09.
Sironen, T., Vaheri, A., Plyusnin, A., 2005. Phylogenetic evidence for the distinction of Saaremaa and Dobrava hantaviruses. Virol. J. 2, 90. https://doi.org/10.1186/1743-422X-2-90.
Song, J.W., Gligic, A., Yanagihara, R., 2002. Identification of Tula hantavirus in Pitymys subterraneus captured in the Cacak region of Serbia-Yugoslavia. Int. J. Infect. Dis. 6, 31–36. https://doi.org/10.1016/S1201-9712(02)90133-5.
Song, J.W., Baek, L.J., Song, K.J., Skrok, A., Markowski, J., Bratosiewicz-Wasik, J., Kordek, R., Liberski, P.P., Yanagihara, R., 2004. Characterization of Tula virus from common voles (*Microtus arvalis*) in Poland: evidence for geographic-specific phylogenetic clustering. Virus Genes 29, 239–247. https://doi.org/10.1023/B:VIRU.0000036384.50102.cf.
Tamura, K., Peterson, D., Peterson, N., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. https://doi.org/10.1093/molbev/mst197.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680. https://doi.org/10.1007/978-1-4020-6754-9_3188.
Tkachenko, E.A., Witkowski, P.T., Radosa, L., Dzagurova, T.K., Okulova, N.M., Yunicheva, Y.V., Vasilenko, L., Morozov, V.G., Malkin, G.A., Krüger, D.H., Klempa, B., 2015. Adler hantavirus, a new genetic variant of Tula virus identified in Major's pine voles (*Microtus majori*) sampled in southern European Russia. Infect. Genet. Evol. 29, 156–163. https://doi.org/10.1016/j.meegid.2014.11.018.
Tougard, C., Montuire, S., Volobouev, V., Markova, E., Contet, J., Aniskin, V., Quere, J.P., 2013. Exploring phylogeography and species limits in the Altai vole (Rodentia: Cricetidae). Biol. J. Linnean. Soc. 108 (2), 434–452. https://doi.org/10.1111/j.1095-8312.2012.02034.x.
Vaheri, A., Henttonen, H., Voutilainen, L., Mustonen, J., Sironen, T., Vapalahti, O., 2013. Hantavirus infections Europe and their impact on public health. Rev. Med. Virol. 23, 35–49. https://doi.org/10.1002/rmv.1722.
Vapalahti, O., Lundkvist, A., Kukkonen, S.K., Cheng, Y., Gilljam, M., Kanerva, M., Manni, T., Pejcoch, M., Niemimaa, J., Kaikusalo, A., Henttonen, H., Vaheri, A., Plyusnin, A., 1996. Isolation and characterization of Tula virus, a distinct serotype in the genus Hantavirus,family Bunyaviridae. J. Gen. Virol. 77, 3063–3067. https://doi.org/10.1099/0022-1317-77-12-3063.
Vapalahti, O., Mustonen, J., Lundkvist, A., Henttonen, H., Plyusnin, A., Vaheri, A., 2003. Hantavirus infections in Europe. Lancet Infect. Dis. 3, 653–661. https://doi.org/10.1016/S1473-3099(03)00774-6.
Whitehouse, C.A., Kuhn, J.H., Wada, J., Ergunay, K., 2015. Family Bunyaviridae. In: Shapshak, P., Sinnott, J.T., Somboonwit, C., Kuhn, J.H. (Eds.), Global Virology I: Identifying and Investigating Viral Diseases. Springer, New York, pp. 199–246.
Yiğit, N., Çolak, E., Sözen, M., Karataş, A., 2006. Rodents of Türkiye: Türkiye Kemiricileri. Meteksan Yayınevi, Ankara.
Zelena, H., Mrázek, J., Kuhn, T., 2013. Tula hantavirus infection in immunocompromised host, Czech Republic. Emerg. Infect. Dis. 19, 1873–1875. https://doi.org/10.3201/eid1911.130421.