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SHORT REPORT

Field vole-associated Traemmersee hantavirus from Germany represents a novel hantavirus species

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Abstract

Vole-associated hantaviruses occur in the Old and New World. Tula orthohantavirus (TULV) is widely distributed throughout the European continent in its reservoir, the common vole (*Microtus arvalis*), but the virus was also frequently detected in field voles (*Microtus agrestis*) and other vole species. TULV and common voles are absent from Great Britain. However, field voles there harbor Tatenale and Kielder hantaviruses. Here we screened 126 field voles and 13 common voles from Brandenburg, Germany, for hantavirus infections. One common vole and four field voles were anti-TULV antibody and/or TULV RNA positive. In one additional, seropositive field vole a novel hantavirus sequence was detected. The partial S and L segment nucleotide sequences were only 61.1% and 75.6% identical to sympatrically occurring TULV sequences, but showed highest similarity of approximately 80% to British Tatenale and Kielder hantaviruses. Subsequent determination of the entire nucleocapsid (N), glycoprotein (GPC), and RNA-dependent RNA polymerase encoding sequences and determination of the pairwise evolutionary distance (PED) value for the concatenated N and GPC amino acid sequences confirmed a novel orthohantavirus species, tentatively named Traemmersee orthohantavirus. The identification of this novel hantavirus in a field vole from eastern Germany underlines the necessity of a large-scale, broad geographical hantavirus screening of voles to understand evolutionary processes of virus–host associations and host switches.

Keywords Tula orthohantavirus · Tatenale · Hantavirus species · Germany · Field vole · *Microtus agrestis*

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Introduction

Hantaviruses (order *Bunyavirales*, family *Hantaviridae*) have been identified in various small mammal reservoirs, including murine and cricetid rodents, shrews, moles, and bats [1]. Rodent-borne hantaviruses are distributed worldwide and can cause hemorrhagic fever with renal syndrome (HFRS) in humans in the Old World [2, 3]. The hantavirus genome is divided into three segments. The large (L) segment encodes the viral RNA-dependent RNA polymerase (RdRP). The medium (M) segment encodes the glycoprotein precursor (GPC) that is co-translationally cleaved into the amino terminal Gn and the carboxy-terminal Gc parts. The small (S) segment encodes the structural nucleocapsid (N) protein [4]. The S segment of Cricetidae-associated hantaviruses encodes in an overlapping open reading frame (ORF) a short putative nonstructural protein (NSs) that functions as an interferon antagonist [5].

There is a high diversity of vole-borne hantaviruses in the Old and New World. Sin Nombre orthohantavirus (SNV) strain Convict Creek, El Moro Canyon orthohantavirus (EMCV), Isla Vista hantavirus (ISLAV), and Prospect Hill orthohantavirus (PHV) have been associated with the California vole (*Microtus californicus*), montane vole (*Microtus montanus*), prairie vole (*Microtus ochrogaster*), and meadow vole *(Microtus pennsylvanicus*) reservoirs in the New World [6–11]. Several vole-borne hantaviruses are distributed in Asia and mainland Europe including Khabarovsk orthohantavirus (KHAV) in reed vole (*Microtus fortis*), Maximowicz's vole (*Microtus maximowiczii*), and tundra vole (*Microtus oeconomus*), KHAV strain Topografov in reed vole, Fusong orthohantavirus (FUSV) in reed vole, and Yakeshi orthohantavirus in Maximowicz's vole [11–13].

Tula orthohantavirus (TULV) has a broad geographical distribution that ranges from France in the west to Russia in the northeast and Turkey in the south [14–20]. In depth analyses of TULV and its rodent hosts in Europe have recently led to the identification of independently evolving TULV clades associated with the evolutionary lineages Central (CEN) or Eastern (EST) in the common vole (*Microtus arvalis*) [19]. Within each of these host lineages, two deeply divergent TULV clades occur named after their nonoverlapping geographical ranges TULV Central North (CEN.N) and Central South (CEN.S) or Eastern North (EST.N) and Eastern South (EST.S) [18, 20]. TULV has also been detected in other vole species, i.e., field vole (*Microtus agrestis*), narrow-headed vole (*Microtus gregalis*), East-European vole (*Microtus levis* formerly *Microtus rossiaemeridionalis*), Altai vole (*Microtus obscurus*), European pine vole (*Microtus subterraneus*), Major's pine vole (*Microtus majori*), and water vole (*Arvicola* spp.) [12–15, 17, 21, 22]. However, the long-term evolution of TULV appears to be associated with the common vole as suggested by the isolation-by-distance (IBD) relationship between virus strains across Europe that is driven by the genetic diversity of TULV found in this reservoir species [20].

Currently, little is known about the role of the field vole as hantavirus reservoir. TULV RNA detection in field voles is discussed as a result of spillover infection from sympatric common vole reservoir [17, 18]. Field voles from the British Isles were found to be a reservoir for Tatenale hantavirus, a virus only distantly related to TULV [23]. A related sequence was detected more recently in field voles from Kielder Forest in England, 230 km from the area where Tatenale hantavirus was initially detected [24]. To test for the potential role of field voles as reservoir of TULV and its host specificity in sympatry, we collected field voles and common voles in Brandenburg, eastern Germany, including regions where TULV was previously almost exclusively detected in field voles [17].

A total of 126 field voles and 13 common voles were collected in 2006, 2007, 2008, and 2018 at four trapping sites in Brandenburg (Fig. 1a) and dissected according to the standard protocol [25]. Enzyme-linked immunosorbent assay (ELISA) screening of chest cavity fluids (CCF) was performed in parallel with *Saccharomyces cerevisiae*-produced N proteins of two TULV strains, strain Thuringia [26], and strain Moravia [19, 22, 27]. Here, four of 126 field voles and one of 13 common voles from two trapping sites were detected in both ELISAs as seropositive (Table 1).

Screening of lung tissue-derived RNA by conventional reverse transcription-polymerase chain reaction (RT-PCR) assays targeting the S segment [22], M segment [28], and L segment [29] resulted in specific amplification products in three of 126 field voles and one of 13 common voles (Table 1). The common vole and one field vole were anti-TULV antibody and TULV RNA positive. Two field voles were exclusively anti-TULV positive, but RT-PCR negative, whereas an additional field vole was only TULV RNA positive (Table S1).

For phylogenetic analysis, additional 12 S, 31 M, and 32 L segment TULV sequences were generated for TULV strains that were identified in a previous study (Ref. [18]; Table S2). Direct sequencing of the S and M segment RT-PCR products of three voles of the current study resulted in the identification of sequences from the TULV-CEN.N clade in two field voles collected at Stadtsee and in one common vole from Traemmersee (Fig. S1A and B). The novel partial L segment sequences also clustered within the TULV-CEN.N clade (data not shown). Cytochrome *b* (*cytb*) analysis revealed that the TULV RNA-positive common vole belongs to the evolutionary lineage Central in this species

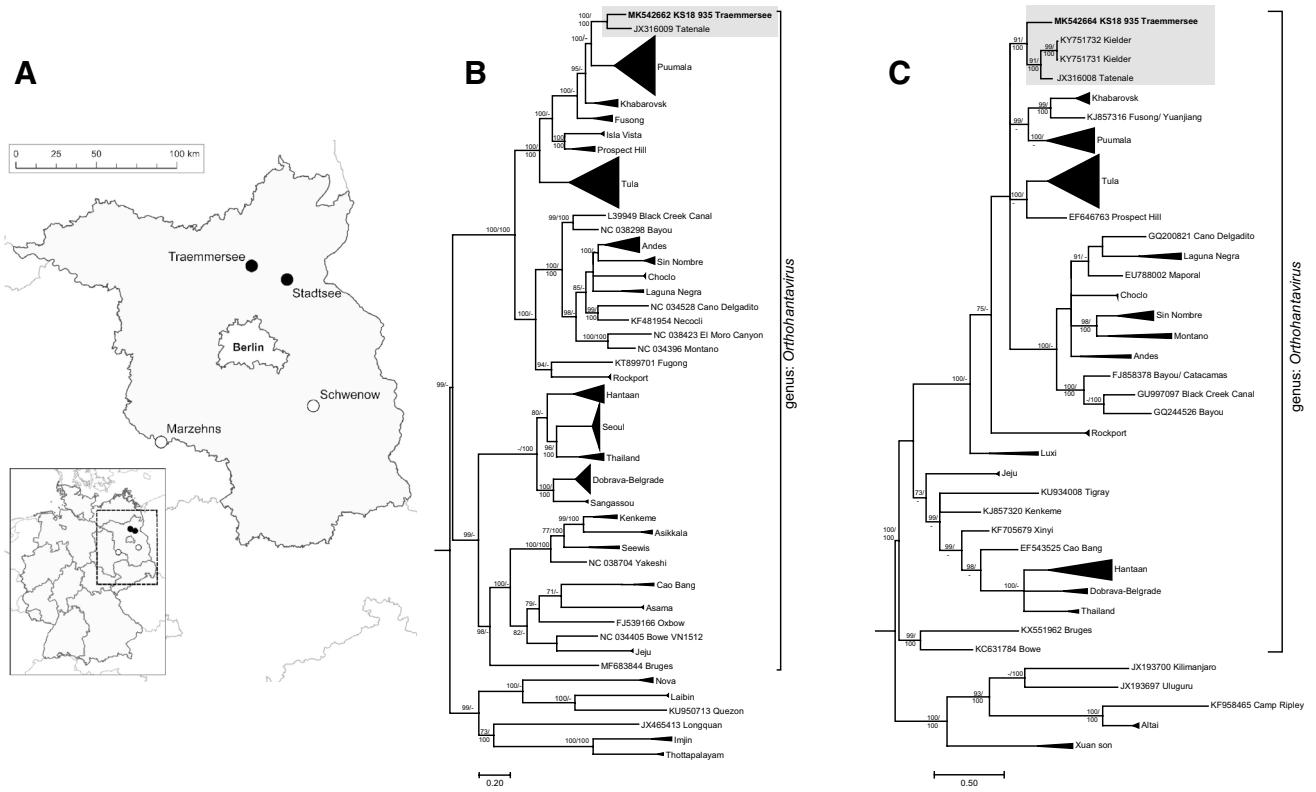

Fig. 1 Map of vole trapping sites and phylogenetic trees of partial S and L segment sequences of hantaviruses including the new Traemmersee orthohantavirus and the British Tatenale and Kielder hantaviruses. **a** Map of the trapping sites of field voles (*Microtus agrestis*) and common voles (*Microtus arvalis*) in Brandenburg, eastern Germany. Trapping sites of hantavirus-RNA-positive field voles and common voles are indicated by filled circles. **b** Consensus phylogenetic tree of the partial S segment sequences (alignment length 393 nucleotides (nt), positions 622–1003, counting according to Tula orthohantavirus (TULV) S segment, accession number NC_005227). **c** Consensus phylogenetic tree of partial L segment sequences (alignment length 333 nt, positions 2983–3309, counting according to TULV L segment, accession number NC_005226). Phylogenetic trees for partial S and L segment sequences were constructed because for Tatenale and Kielder hantaviruses only partial sequences are available. The consensus trees are based on Bayesian analyses with 15,000,000 generations and a burn-in phase of 25%, and maximum-likelihood analyses, with 1000 bootstraps and 50% cutoff using the general time reversible (GTR) substitution model with invariant sites and a gamma distributed shape parameter for both algorithms. Posterior probabilities in percent from Bayesian analyses are given before the slash and bootstrap values are given after the slash for major nodes when they exceeded 70. The tree reconstructions were done via CIPRES [37]. Alignments were constructed under Bioedit (V7.2.3.) [38] using the Clustal W Multiple Alignment algorithm implemented in the program. Names in bold indicate newly generated sequences (MK542662 and MK542664) and field vole viruses clustering with Traemmersee virus are highlighted by a gray box. Triangles indicate compressed branches. Additional accession numbers are listed in Table S6

which is consistent with large-scale phylogeographic patterns (Refs. [19, 30]; Table S1, Fig. S2A).

Interestingly, S and L segment sequences from a single seropositive field vole from Traemmersee were highly divergent to TULV, but similar to the British field vole-associated Tatenale and Kielder hantaviruses (Fig. 1b, c, Table S3). In addition, RT-PCR amplified partial GPC-encoding sequence was also highly divergent from TULV M segment sequences (identity of 75–80%; Fig. S1B); M segment sequences from Tatenale and Kielder hantaviruses are not available so far.

The complete coding sequences of S, M, and L segments were generated by a primer-walking approach (for primers used see Table S4). The encoded N protein, GPC, and RdRP are 433, 1148, and 2154 amino acids in length, respectively. A moderate similarity to TULV and a higher similarity to the sequences of other vole-borne hantaviruses were identified by pairwise comparison of the nucleotide and amino acid sequences as well as in the phylogenetic trees (Table S5 and Fig. S3A–F). A 270 nucleotide-long NSs ORF overlapping the N ORF was identified; the amino terminal region of the putative NSs protein is similar to that of the majority of vole-borne hantaviruses, but differs to the amino-terminally extended NSs proteins of KHAV and FUSV (data not shown).

The pairwise evolutionary distance (PED) values of the concatenated N protein and GPC of Traemmersee virus and KHAV, FUSV, Puumala orthohantavirus (PUUV), PHV, and TULV vary between 0.14 and 0.66 (Table S5). These values

Table 1 Results of the serological and RT-PCR investigations of field voles (*Microtus agrestis*) and common voles (*Microtus arvalis*) from four trapping sites in eastern Germany

Trapping site (see Fig. 1a)	Trapping year	Species	Total number of voles trapped	Results (number positive/total number tested)			Virus (lineage)
				IgG ELISA TULV EST.S (Moravia) N protein	IgG ELISA TULV-CEN.N (Thuringia[a]) N protein	S segment RT-PCR	
Marzehns	2006	*M. agrestis*	9	0/9	0/9	0/9	
	2007	*M. agrestis*	33	0/33	0/33	0/33	
		M. arvalis	7	0/7	0/7	0/7	
Schwenow	2006	*M. agrestis*	5	0/5	0/5	0/5	
	2008	*M. agrestis*	3	0/3	0/3	0/3	
		M. arvalis	1	0/1	0/1	0/1	
Stadtsee	2006	*M. agrestis*	12	0/12	0/12	0/12	
	2007	*M. agrestis*	14	0/14	0/14	0/14	
	2008	*M. agrestis*	39	3/39	3/39	2/39	TULV (CEN.N, Central North)
Traemmersee	2008	*M. agrestis*	8	1/8	1/8	1/8	Traemmersee virus
		M. arvalis	5	1/ 5	1/5	1/5	TULV (CEN.N, Central North)
	2018	*M. agrestis*	3	0/3	0/3	0/3	
Total		*M. agrestis*	126	4/126	4/126	3/126	
		M. arvalis	13	1/13	1/13	1/13	
		all *Microtus*	139	5/139	5/139	4/139	

[a]Amino-terminally his-tagged nucleocapsid (N) protein of Tula orthohantavirus (TULV) strain from Thuringia was produced in yeast *Saccharomyces cerevisiae* and purified by nickel-chelate affinity chromatography under denaturing conditions as described previously [27]

were higher than the cutoff value (0.1) that was defined by the International Committee on Taxonomy of Viruses (ICTV) for a hantavirus species using *D*iv*E*rsity p*A*rtitioning by hie*R*archical *C*lustering (DEmARC) [31]. According to the criteria of the ICTV [31], this suggests a novel virus species that was tentatively named according to the trapping site of the field vole "Traemmersee orthohantavirus" (TRAV). The definition of a novel virus species is also supported by the lack of evidence for recombination of the entire S, M, and L segment nucleotide sequences of TRAV and corresponding reference sequences in SimPlot and RDP4 analyses (Refs. [32, 33]; data not shown). Comparison of the partial N protein sequence of Tatenale hantavirus (131 aa) and TRAV revealed a PED value of 0.0395, which may indicate that Tatenale hantavirus and TRAV represent a single orthohantavirus species.

The paucity of available data on British Tatenale and Kielder hantaviruses and TRAV from other locations prevents at present conclusive analyses of their evolutionary history. However, field voles colonized the British Isles only after the last glaciation less than 15,000 years ago [34–36] which suggests a continental origin of the ancestor of these British hantaviruses. At present, we can only speculate that the evolutionary history of these hantaviruses might be associated with the history of the Western *cytb* lineage in the field vole because the British Isles were colonized by this lineage [36] and the field vole population at the sampling location of TRAV harbors both the Central and Western lineages.

In conclusion, we identified a novel orthohantavirus species in Germany which is most similar to British hantaviruses detected in the same vole host species. More hantavirus data based on large-scale geographical screening will be necessary to understand the evolutionary history of this system better. However, detailed analyses of TULV clades and evolutionary lineages in the common vole have recently demonstrated that speciation processes in hantaviruses can be triggered by evolutionary divergence in their hosts and may even outrun host evolution [19]. It seems thus appropriate to explicitly consider not only the presumed reservoir host but also related species as potential hosts for a better understanding of the role of host association and host switches in the evolution of hantaviruses.

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Author contributions RGU and GH designed the study. MW collected all voles. KJ performed all molecular and serological investigations including sequence determination and analyses and contributed to the

generation of the N antigen of TULV strain Thuringia. MH performed RDP4 and SimPlot analyses. AS and RP contributed to the generation of both TULV N antigens. SD, RR, and GH supervised the sequence analyses. RR performed the PED determination. KJ, GH, and RGU wrote the manuscript draft. All authors contributed to the final version of the manuscript and approved it.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The collection of voles was performed by the local forestry institutions during the vole monitoring as part of their pest control measures.

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