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Short communication

First molecular evidence of Tula hantavirus in *Microtus* voles in Slovenia

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ABSTRACT

Different *Microtus* species, present in a worldwide range habitat populating North America, Europe, Asia, and few other species have been recognized previously as a hantavirus reservoir. Tula hantavirus was first reported in *Microtus arvalis* and *Microtus rossiaemeridionalis* from Central Russia and later discovered in several European countries. Using molecular techniques we have demonstrated the presence of Tula hantavirus in three different *Microtus* species in Slovenia. Phylogenetic analyses of partial S segment placed Slovenian strains in the same genetic lineage as Austrian and Croatian strains.

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Hantaviruses constitute a separate genus within the *Bunyaviridae* family. The most important difference between *Hantavirus* genus and other genera in the same family is the fact, that hantaviruses do not have an arthropod vector. This unique feature of hantaviruses enabled them to develop a tight bond between the virus and their specific rodent or insectivore host worldwide. In Europe, several hantaviruses have been demonstrated to circulate in nature: Puumala (PUUV), Dobrava (DOBV), Saaremaa (SAAV), Seoul (SEOV) and Tula (TULV) (Heyman et al., 2004; Lundkvist and Plyusnin, 2002; Nemirov et al., 1999; Plyusnin et al., 1994). They are primarily associated with the bank vole, *Myodes glareolus*, yellow necked mouse, *Apodemus flavicollis*, striped field mouse, *Apodemus agrarius*, Norway rat, *Rattus norvegicus*, and common vole, *Microtus arvalis*, respectively (Vapalahti et al., 2003).

Wide distribution of hantaviruses across Europe, Asia and America is feasible on behalf of their coevolution with rodent reservoir. Although hantavirus spillover infections in other rodent hosts have been demonstrated, the virus is still believed to be associated with a single rodent species in which it establishes persistent infection (Childs et al., 1987; Plyusnin and Morzunov, 2000). The genus *Microtus* is present in a worldwide range habitat, populating the North America, Europe and Asia (Nowak, 1999) and several species have already been recognized as a hantavirus reservoir. Namely, several hantavirus serotypes have been isolated from *Microtus* spp. not only in Europe: Khabarousk and Vladivostok virus isolated from *Microtus fortis* (Horling et al., 1996; Kariwa et al., 1999) and TULV in *M. arvalis* and *Microtus rossiaemeridionalis* (Plyusnin et al., 1994), but also in America: Prospect Hill virus in *M. pennsylvanicus* (Lee et al., 1982), Isla Vista virus in *M. californicus* (Song et al., 1995) and Bloodland Lake virus in *M. ochrogaster* (Hjelle et al., 1995) and Asia: several hantavirus strains isolated from *M. fortis* and *M. maximowiczii* (Zou et al., 2008). Despite some evidence of possible human infection with TULV (Klempa et al., 2003; Vapalahti et al., 1996), currently no clinical disease has been clearly associated with any *Microtus*-carried hantaviruses (Scharninghausen et al., 2002).

TULV was first described in the 1994, when virus sequences were obtained from *M. arvalis* captured in Tula region in Central Russia (Plyusnin et al., 1994). Since then several other countries in Central and Eastern Europe have reported the presence of TULV-infected *M. arvalis* (Bowen et al., 1997; Heyman et al., 2002; Klempa et al., 2003; Plyusnin et al., 1995; Scharninghausen et al., 2002; Sibold et al., 1999; Song et al., 2002, 2004; Jakab et al., 2008; Plyusnina et al., 2007, 2008a; Reusken et al., 2008). *M. arvalis* are believed to be natural reservoir of TULV. However, two reports demonstrated the presence of TULV in *M. agrestis* in Croatia (Scharninghausen et al., 2002) and a *Microtus subterraneus* (re-classified from *Pitymys subterraneus*) in Serbia (Song et al., 2002).

Slovenia's neighboring countries have all reported the presence of TULV-infected voles and Slovenia is a well known endemic region for different hantaviruses: DOBV, PUUV, SAAV which have been detected in rodent reservoir and DOBV, PUUV also in patients (Avsic-Zupanc et al., 2000, 2007). Therefore we have tested *Microtus* spp. trapped in different regions in Slovenia as possible hantavirus reservoir. In addition to detecting TULV in common voles in different regions of Slovenia, we have also demonstrated the presence of the virus in the field vole and the European pine vole. To our best knowledge this is the first description of the same hantavirus in three different species within a single country.

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Rodent trapping has been conducted biannually, in spring and autumn, in different HFRS regions in Slovenia since 1990. Sherman type live traps were placed strategically to caught *Apodemus* sp. and *Myodes glareolus* preferably, as they are a recognized hantavirus reservoirs in Slovenia. Each animal caught was identified by the professional taxonomist and animal's weight, size and sex were recorded. Following the sample collection, animals were sacrificed and their internal organs (heart, lungs, liver, spleen, kidneys and urine bladder) were collected and stored in cryovials at −80 °C. Blood was collected by cardiac puncture and centrifuged for 5 min at 2000 rpm. Serum was separated from the blood clot and both were stored in cryovials at −20 °C. Apart from *Apodemus* sp. and *Myodes* sp., 75 other rodent and shrew species have been captured. The *Microtus* species classification was additionally confirmed by using cytochrome *b* as a phylogenetic marker (data available upon request).

Total RNA was extracted using TRIZOL Reagent (Invitrogen Life Technologies™) from lung samples of two water voles (*Arvicola terrestris*), 8 field voles (*Microtus agrestis*), 15 common voles (*M. arvalis*), 5 snow voles (*Microtus nivalis*), 3 Liechtenstein's pine voles (*Microtus liechtensteini*), 1 European pine vole (*M. subterraneus*), 4 bicolured white-toothed shrews (*Crocidura leucodon*), 4 lesser white-toothed shrews (*Crocidura suaveolens*), 12 Mediterranean water shrews (*Neomys anomalus*), 8 Eurasian water shrews (*Neomys fodiens*), 1 alpine shrew (*Sorex alpinus*) and 12 common shrews (*Sorex araneus*). Tissue samples for RNA isolation were thawed in a bio-safety chamber. 1 mm^3 of tissue core was transferred into a sterile tube and PBS was added up to 200 μL. Tissue was homogenized using an automated homogenizator. Extracted RNA was eluted in Nuclease-Free Water (Promega) and analyzed using real time RT-PCR assay specific for TULV (Kramski et al., 2007). Out of 75 tested voles and shrews, only voles were positive: 5 *M. arvalis* (5/15, 33%), 1 *M. agrestis* (1/8, 12.5%) and 1 *M. subterraneus* (1/1, 100%). In order to obtain TULV sequences from positive samples, nested RT-PCR assay was preformed amplifying 307 nt long region of the S segment. The protocol used has been described elsewhere (Bowen et al., 1997). With the exception of one lung samples of *M. arvalis*, we have amplified and characterized partial S segment sequences from all other positive samples. To obtain additional genetic information of TULV in Slovenia, we have amplified a more conservative region of TULV genome using degenerated primers, described by Klempa et al. (2006). Despite using this broad spectrum nested RT-PCR assay the detection was successful in only four samples, yielding 402 nt long fragments of the TULV L segment. When comparing both nested RT-PCR assays and real time RT-PCR assay specific for TULV we have observed that the most sensitive method for detecting TULV from natural specimen is the real time RT-PCR assay.

All samples that demonstrated the positive reaction in one or both nested RT-PCR assays were sequenced on both strands by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM, PE Applied Biosystems, CA, USA). The obtained sequences were analyzed with the modules of the Lasergene 5.0 software package (Dnastar, Madison, WI, USA). Sequence alignments were performed using the CLUSTAL W algorithm. Phylogenetic relationships of TULV from Slovenian strains and other sequences in the GenBank were established with the software package TREECON. Phylogenetic tree was constructed from the sequence analysis of a partial region of the S segment by neighbor-joining method with Kimura 80 evolutionary model for nucleotide substitutions. Support for the tree nodes was calculated with 2000 bootstrap replicates. Representative sequences of the novel Slovenian TULV strains: TULV/Crna vas/Ma225/95, TULV/Zizki/Ma69/96, TULV/Griblje/Ma57/01, TULV/Sred ob Dravi/Ms51/97, TULV/Hodos/Ma99/99, TULV/Sestrze/Mag98/02 described here were deposited in GenBank under accession numbers: FJ495093–FJ495102.

To gain new information on TULV reservoir in Slovenia in comparison to neighboring countries, different regions in the country and voles and shrew hosts were selected for the study. Partial S segment sequences (306 nt) were recovered from six samples isolated from three different *Microtus* species in Slovenia. Nucleotide sequence analysis revealed the presence of TULV in all of them, with the 86.9–100% identity among the strains. However, deduced amino acid sequences (101 AA) of the encoded part of the nucleocapsid protein were identical. Partial L segment (402 nt) of TULV sequences, recovered from four samples, represented three different *Microtus* species (2 *M. arvalis*, 1 *M. agrestis* and 1 *M. subterraneous*). Sequence distances showed higher diversity ranging from 86.1% to 90.3%. Deduced amino acid sequences of the encoded part of the viral polymerase were

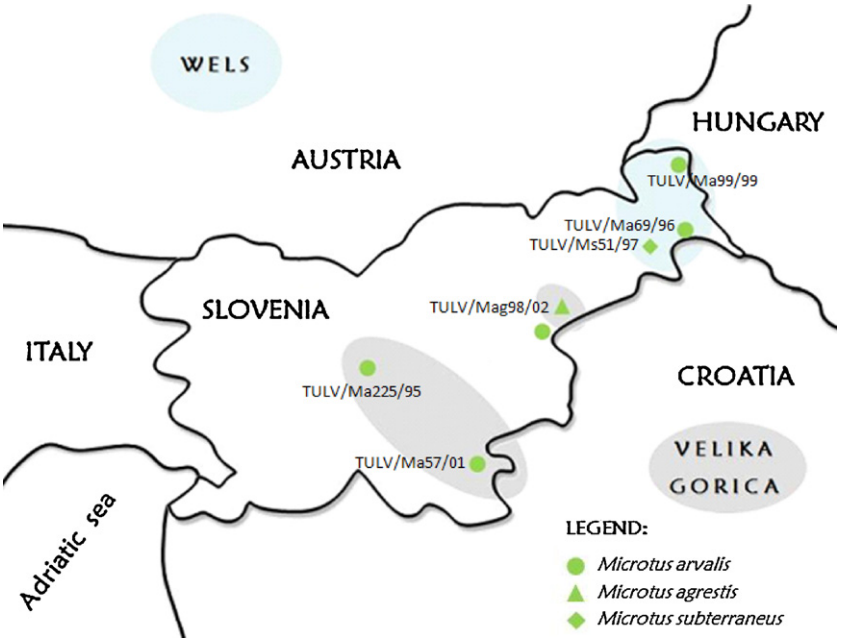

Fig. 1. Map of Slovenia showing trapping sites of TULV-infected voles and neighboring countries trapping sites belonging to the same monophyletic lineage of TULV.

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