

## Tula hantavirus in Belgium

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### SUMMARY

European common voles (*Microtus arvalis*), captured in Belgium in 1999, were proven by molecular as well as by serological techniques to be infected with Tula hantavirus (TULV). This is the first evidence for the presence of TULV in this country. No indication of spill-over infections of Puumala virus, known to be highly endemic among bank voles (*Clethrionomys glareolus*) within the same geographical regions as the trapped TULV-infected common voles, was observed. Together with previous reports on the circulation of TULV in eastern/central Europe, this finding suggests a more wide-spread circulation of this hantavirus serotype throughout the continent.

### INTRODUCTION

Hantaviruses are the aetiological agents of haemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and hantavirus pulmonary syndrome (HPS) on the American continent. The various serotypes are maintained in specific rodent host species, which belong to the family Muridae, subfamilies Murinae, Arvicolinae and Sigmodontinae [1, 2]. Murinae rodents are the reservoir hosts for Hantaan (HTNV), Seoul (SEOV) and Dobrava (DOBV) hantaviruses, which all cause severe forms of HFRS in Asia and Europe [3, 4]. Arvicolinae rodents host Puumala (PUUV), Tula (TULV), Topografov (TOPV) and Khabarovsk (KBRV) hantaviruses mainly in the temperate zone of Eurasia, and Prospect Hill (PHV), Isla Vista (ILVV) and Blood Land Lake (BLLV)

hantaviruses in North America [1, 5]. Of all described Arvicolinae-associated hantaviruses only PUUV has been proven as a human pathogen. Sin Nombre-like hantaviruses are the causative agents for HPS in the Americas and are carried by Sigmodontinae rodents [6]. Transmission of the viruses to man occurs through infected animal excreta, i.e. urine, faeces and saliva. In Europe, so far only PUUV and DOBV have been associated with human disease. PUUV, carried by bank voles (*Clethrionomys glareolus*), is responsible for a milder form of HFRS, called nephropathia epidemica [7]. In most European countries, PUUV-attributed infections have been described and occur regularly with cyclic outbreak periods.

TULV, carried by European common voles (*Microtus arvalis*), was first described in 1994 [8] and is considered to be non-pathogenic to man; only one TULV-positive serum was found after screening of

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hundreds of human sera from central Russia and Czech Republic [9]. *Microtus arvalis* is common throughout the Palaearctic region, except in Great Britain, Scandinavia, Siberia, the Balkans and the Mediterranean regions [10, 11]. On the Orkney Islands and Guernsey, they exist as a relic form [12]. An isolated, probably introduced, population has been found on the Spitzbergen Islands [13].

So far, TULV has only been detected in Eastern and Central Europe [8, 9, 14, 15] but the more extended geographical distribution of its host led us to suspect that this hantavirus serotype may also be prevalent in Belgium. The present study, undertaken in 1999, was designed to test this assumption.

The year 1999 was expected to be a hantavirus-epidemic year in Belgium as a 3-year epidemic cycle was already described and 1996 showed a significant increase of human cases [16]. Although each hantavirus is associated predominantly with one host rodent, we decided to check also if spill-over infection of PUUV from bank voles to common voles might have taken place. Bank voles live mainly within woods but may occasionally also be found in grasslands up to 30 metres from the wood-edges while *Microtus arvalis* exclusively lives in grasslands [17]. Both species may thus interact either directly by physical contact or indirectly through their excreta.

Common voles were trapped on 10 different locations in the country in order to assess seroprevalence, geographical distribution and genetic variation of hantaviruses in the different populations.

## METHODS

### Capture of rodents

Capture sessions were performed from May to September 1999 at 10 trapping sites distributed all over Belgium. The trapping sites were located in broad leaf tree forest, and mixed pine and broad leaf tree forest, both with a dense scrub wood or ground vegetation, to grasslands on the border of woods. Apart from other rodent species, 65 *M. arvalis* were captured in South and East Belgium.

Capture sessions were undertaken on two or three consecutive nights; rodents were caught with Longworth traps (Bolton Inc., UK) containing hydrophobic cotton as nesting material and rolled oats as bait or with Ugglan vole live traps (Grahnb, Sweden) baited with apple and peanut butter. The traps were placed 10 metres apart on rows and left during the

night. The following morning, the traps were inspected for the presence of rodents. Captured rodents were anaesthetized with isoflurane (Forène, Abbott, Abbott Park, IL, USA). Body weight, body-, tail- and back paw length were measured and species and sex were determined. The animals were sacrificed and dissected; blood was obtained by cardiac puncture and lungs and kidneys were removed. Whole blood samples were centrifuged and the serum samples were cooled immediately after the dissection session. Lungs and kidneys were stored in RNAlater (Ambion Inc, Austin, Texas, USA) and treated following the manufacturers specifications. The traps were cleaned, baited again and put out for the next night on the same place.

### Serological screening

Initial screening was performed on the samples obtained from trapped *M. arvalis* by applying standard ELISA techniques, using native inactivated viral antigens (PUUV – strain CG-1820, DOBV – strain Slovenia, HTNV – strain 76-118) [18] and recombinant antigens (PUUV – strain Kazan, and DOBV – strain Slovenia) (C. de Carvalho Nicacio and Å. Lundkvist, unpublished), for detection of IgG antibodies. In brief, 96-well microtitre plates were coated with antigen and incubated overnight at 4 °C. Unsaturated binding sites were blocked with 3% BSA (Sigma, St Louis, MO) in PBS buffer, followed by incubation of rodent sera diluted 1 in 200 at 37 °C for 1 h. Peroxidase- (Sigma) or alkaline-phosphatase-labelled (Jackson, West Grove, PE) anti-mouse IgG antisera were incubated for 1 h. Subsequently, TMB or p-nitrophenyl substrates (Sigma) were added and, after colour development, the reaction was stopped with 2M H<sub>2</sub>SO<sub>4</sub> (for TMB substrate). The optical density (O.D.) was determined at 450 nm or 405 nm against a reference wavelength of 620 nm.

### Immunoblotting

ELISA positive and borderline reactive samples were analysed by immunoblotting essentially as described previously [19]. Briefly, native hantavirus antigens were applied to 4–20% SDS-polyacrylamide gels (Bio-Rad, CA, USA) and transferred electrophoretically to nitrocellulose filters. Filters were pre-adsorbed and incubated overnight at 4 °C with serum samples diluted 1 in 400. After washing, the filters

were incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat antibodies to mouse IgG (H+L) (Jackson). NBT/BCIP (Sigma) was used as substrate.

### Focus reduction neutralization tests (FRNT)

Hantavirus antibody-positive sera were confirmed and serotyped by FRNT as previously described [20]. Briefly, samples were serially diluted and mixed with an equal volume containing 30–70 focus-forming units (FFU) of virus per 100  $\mu$ l. The mixtures were incubated for 1 h and inoculated into wells of 6-well plates containing confluent Vero E6 cell monolayers. After adsorption for 1 h, the wells were overlaid with agarose and incubated for 7–13 days. Hantavirus-specific polyclonal antisera, followed by peroxidase-labelled goat antibodies and TMB substrate, were used for detection of virus-infected cells. An 80% reduction in the number of foci was used as the criterion for virus neutralization titres.

### RT-PCR, cloning and partial sequencing

RNA from lungs of dissected *M. arvalis* was extracted using a guanidine isothiocyanate, phenol containing solution (RNA NOW, Biogentex, Seabrook, TX, USA) according to the manufacturer's instructions. Reverse transcription was performed using First-Strand cDNA synthesis kit (Amersham pharmacia biotech, Uppsala, Sweden) according to the manufacturer's protocol using hantavirus-specific primer 5'TAGTAGTAGAC3'. PCR-amplification of the S segment was performed with primer 5'TTCTGCAGTAGTAGTAGACTCCTTGAAAAG3' [8]. Nested PCR was performed with primers 5'TAGTAGACT-(C/T)CGT(A/G)AA(A/G)AGCTACTA 3' and 5'ATTACATC(A/T)A(A/G)GACATT(C/T)CCATA3' flanking the region nt 29–333. PCR-amplicons were purified and sequenced using a DNA sequencing kit (ABI Prism. PE Biosystems, Foster City) on a 3100 genetic analyser (PE Biosystems).

The PHYLIP program package (Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle) was used to generate 100 bootstrap replicates of the sequence data (Seqboot). Distance matrices were calculated using Kimura's 2 parameter model (Dnadist) and analysed by the Neighbour-joining tree fitting algorithm (Neighbour). The bootstrap support per-

centages of particular branching points were calculated from these trees (Consense). Isla Vista (ILVV) and Topografov (TOPV) hantaviruses (Genebank accession numbers U31534 and AJ011646, respectively) were used as outgroups. For comparison, the following TULV sequences were obtained from the Genebank: strains Lodz-1 (accession number AF063892) and Lodz-2 (AF063897) from Poland, strains D63-98 (AF289821), D17-98 (AF289820), D5-98 (AF289819), and AF164093 (AF164093) from Germany, strains Tula/53Ma/87 (Z30942), Tula/23Ma/87 (Z30945), Tula/175Ma/87 (Z30943), Tula/76Ma/87 [8] (Z30941), and Tula/249Mr/87 (Z30944) from Russia, strain AF017659 from Serbia (AF017659), strains Kosice144/Ma/95 (Y13979), Kosice667/Ma/95 (Y13980), Koziky5276Ma/94 (AJ223601), Koziky5247Ma/94 (AJ223600), Malacky/Ma370/94 (Z68191), Malacky/Ma32/94 (Z48235), Moravia/5294Ma/94 (Z48741), Moravia/5302Ma/94 (Z49915), strain Moravia/5293Ma/94 (Z48574), Moravia/5286Ma/94 (Z48573) from Czech republic [9, 21] and Slovakia [21, 22], strain AF164094 from Croatia (AF164094). The sequence of the TULV strain Switzerland/Ma91/95 is from the unpublished data of F. Saucy and A. Plyusnin.

## RESULTS

Sixty-five *M. arvalis* serum samples were initially screened by ELISA for the presence of hantavirus antibodies. Five (7.7%) showed clear IgG-reactivity for PUUV by ELISA, while five other samples showed borderline reactivity to at least one of the included antigens (Table 1). The five ELISA-positive samples and five ELISA-borderline samples were further assayed by immunoblotting; four of the five ELISA-positive samples reacted with native PUUV, while all four samples examined with native TULV reacted with this virus (Table 1). Lung samples from all five hantavirus serum-positive *M. arvalis* were also found positive by nested RT-PCR. The cloned fragment (nt 1–542) of sample #9 showed high level of identity with known sequences of TULV, ranging from 87–90% depending on the TULV strain selected for the comparison, thus confirming the virus as TULV. Sequence homologies to PHV, PUUV, KBRV, TOPV, DOBV, HTNV and SEOV were 80%, 78%, 78%, 77%, 62%, 62%, and 61%, respectively. The fragments from the nested PCR-products of samples #3, 6, 7 and 9 showed 99–100% identity with the cloned fragment of sample #9. The fragments of

Table 1. *Hantavirus IgG ELISA, Western Blot, RT-PCR/Sequence and FRNT results on 10 M. arvalis*

Animal	IgG ELISA			Western Blot		PCR/Seq	Focus reduction neutralization test				
	PUUV*	PUUV†	HTNV†	PUUV	TULV	TULV	HTNV	SEOV	DOBV	PUUV	TULV
1	2·643‡	1·615	1·133	+	+	+ / +	40	40	40	80	1280
2	0·008	0·097	0·024	–	–	NT	< 40	< 40	< 40	< 40	< 40
3	1·114	0·112	0·030	+	+	+ / +	< 40	40	< 40	80	1280
4	0·049	0·126	0·023	–	–	NT	< 40	< 40	< 40	< 40	< 40
5	0·122	0·092	0·048	–	–	NT	< 40	< 40	< 40	< 40	< 40
6	NT	0·249	0·059	–	+	+ / +	< 40	< 40	< 40	< 40	80
7	3·042	1·589	0·950	+	NT	+ / +	< 40	80	80	320	5120
8	0·022	0·682	0·220	–	–	NT	< 40	< 40	< 40	< 40	< 40
9	1·079	1·745	1·108	+	+	+ / +	40	40	80	320	1280
10	0·127	0·313	0·278	NT	NT	NT	< 40	< 40	< 40	< 40	< 40

\* Results from ELISA screening based on recombinant PUUV strain Kazan full-length N antigen, CO 0·200.

† Results from ELISA screening based on native inactivated viral antigens PUUV strain CG 18-20 and HTNV 76-118, CO 0·200.

‡ Test to negative OD values.

Western blot: + positive, – negative.

RT-PCR/sequencing: + : positive PCR signal/ + : TULV sequence obtained.

Focus Reduction Neutralization Test: Reciprocal end-point titres (starting from 1:40 dilution).

NT: not tested

samples #3 and #9 were identical as well as the fragments of samples #6 and #7. The corresponding wild TULV strains were designated as TUL/Belgium/Ma6/99, and TUL/Belgium/Ma9/99 (or Belg6 and Belg9, for short). The fragment of sample #1 (TUL/Belgium/Ma1/99, or Belg1 for short) showed 97% identity to the four other Belgian sequences.

On the phylogenetic tree (Fig. 1) three Belgian sequences formed a well-supported cluster. Within this cluster, strains Belg6 and Belg9 were located closer to each other than to the third strain, Belg1. The Belgian cluster seemed to share a common ancestor with TULV strain from Switzerland and one of the German strains (Germ AF1640); however the bootstrap support value for placing them together did not exceed the confidential limit of 70%.

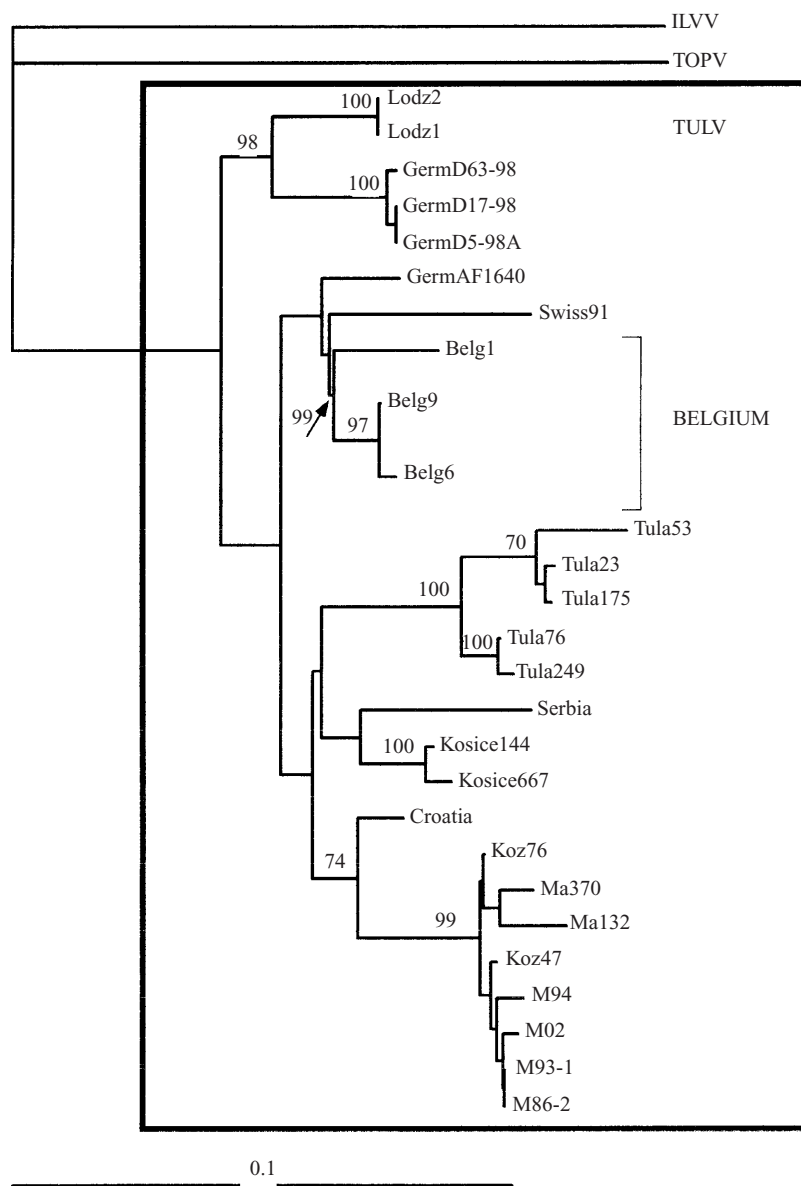
By FRNT, end-point titres against HTNV, SEOV, DOBV, TULV and PUUV were determined. Of the five ELISA-borderline and immunoblot-negative samples, all were found FRNT-negative against all hantaviruses included in the analyses (Table 1). Of the five remaining sera, three sera (no. 1, 3, 7) showed a 16-fold higher end-point titre (titre range: 1280–5120) for TULV than for the other four viruses, one sample (no. 9) showed an at least fourfold higher titre to TULV than to the other viruses, while one sample (no. 6) was found clearly positive for TULV (titre 80) and negative (< 40) for the four other viruses. These

findings confirmed that the five hantavirus antibody-positive rodents were all infected with TULV. No indication of spill-over infections by PUUV, known to be carried by *C. glareolus* in the same geographical regions, was observed.

## DISCUSSION

Although the prevalent hantaviral serotype in western Europe seems to be PUUV [18], we started capture sessions on 10 well defined areas in Belgium in order to obtain more information on the distribution and genetic diversity of hantaviruses circulating in Belgium. The year 1999 exhibited high rodent population densities together with high hantavirus seroprevalence rates [23, Heyman et al. unpublished].

Five (7·7%) of 65 *M. arvalis* were shown to be infected with TULV by RT-PCR and subsequent sequencing of the amplicons. FRNT, which has so far only occasionally been used for demonstrating a specific serotype of hantavirus infection in rodents, confirmed the results of the molecular techniques. Spill-over infections of a certain hantavirus to other than the natural reservoir rodent species have been suggested on several occasions and a number of European rodent species, not known to carry a defined hantavirus, have been reported as hantavirus infected by serological data based on e.g. IFA or



**Fig. 1.** Phylogenetic tree (Neighbour-joining) of TULV based on the nucleocapsid protein encoding sequence of the S segment (nt 29–333). Isla Vista (ILVV) and Topografov (TOPV) hantaviruses are used as outgroups. Only bootstrap support values greater than 70% are shown. The following nicknames for the TULV strains are used: Lodz1 = strain Lodz-1; Lodz2 = strain Lodz-2; GermD63-98 = German isolate D63-98; Germ D17-98 = German isolate D17-98; GermD5-98A = German isolate D5-98A (AF289819); GermAF1640 = German strain AF164093; Swiss91 = strain Switzerland/Ma91/95; Tula53 = strain Tula/53Ma/87; Tula23 = strain Tula/23Ma/87; Tula175 = strain Tula/175Ma/87; Tula76 = strain Tula/76Ma/87; Tula249 = strain Tula/249Mr/87; Serbia = strain AF017659; Kosice144, = strain Kosice144/Ma/95; Kosice667 = strain Kosice667/Ma/95; Croatia = strain AF164094; Koz76 = strain Kosiky5276Ma/94; Ma370 = strain Malacky/Ma370/94; Ma132 = strain Malacky/Ma32/94; Koz47 = strain Koziky5247Ma/94; M94 = strain Moravia/5294Ma/94; M02 = strain Moravia/5302Ma/94; M93-1 = strain Moravia/5293Ma/94; M86-2 = strain Moravia/5286Ma/94.

ELISA. However, the actual serotypes involved in potential spillover events have never been determined for any European hantavirus. To investigate potential spillover/double infections among *M. arvalis*, trapped in geographical areas where PUUV is known to be present (Heyman et al., unpublished data), we applied

an extensive investigation by FRNT, including all hantaviruses known to circulate in Europe or known to cause HFRS. The highly specific reactivity of neutralizing antibodies to TULV, versus all other included hantaviruses, did not indicate any spillover phenomenon among the *M. arvalis* investigated.



Although *M. arvalis* has already been described as the vector for TULV in Russia [8], Czech Republic [9, 14], Slovakia [15], Austria [24], and Switzerland [25], the observation that TULV is circulating also in Belgium, which is to date the westernmost observation of TULV in Europe, supports our hypothesis that this serotype could be present in *M. arvalis* populations throughout Europe. Geographically, the previously described detection sites in eastern/central Europe are more than 1000 kilometres eastwards from the sites where the virus was now found to be present in Belgium.

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