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Characterization of Tula Virus From Common Voles (*Microtus arvalis*) in Poland: Evidence for Geographic-Specific Phylogenetic Clustering*

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Abstract. Tula virus (TULV), a recently identified arvicolid rodent-borne hantavirus, is harbored by the European common vole (*Microtus arvalis*) in Central Russia and the Czech and Slovak Republics. We report the isolation and characterization of this hantavirus from *M. arvalis* captured in Poland, a country where human disease caused by hantaviruses has not been recognized. Of 34 arvicolid rodents (24 *Clethrionomys glareolus*, 9 *M. arvalis*, 1 *Pitymys* sp.) captured in Lodz and Tuszyn, Poland, during June to September 1995, sera from 3 *M. arvalis* and 3 *C. glareolus* contained IgG antibodies to Puumala virus (PUUV), as determined by an indirect immunofluorescent antibody assay. Alignment and comparison of the 1852-nucleotide S segment and a 1676-nucleotide region of the G2 glycoprotein-encoding M segment, amplified from lung tissues of two hantavirus-seropositive *M. arvalis*, revealed 83.9–85.2% and 82.3–83.5% sequence similarity, respectively, with TULV strains from Central Russia and the Czech and Slovak Republics. A > 98% sequence conservation was found at the amino acid level. Phylogenetic analysis indicated that the newly found TULV strains from Poland were closely related to, but distinct from, TULV from elsewhere in Europe.

Key words: *Hantavirus*, *Microtus*, phylogenetic analysis, Poland, rodents

Introduction

Hantaviruses, as members of the family *Bunyaviridae*, genus *Hantavirus*, possess a tripartite, negative-sense, single-stranded RNA genome, designated large (L), medium (M) and small (S), which encode an RNA-dependent RNA polymerase, envelope glycoproteins (G1, G2) and a nucleocapsid protein, respectively [1,2]. Each hantavirus is associated, and appears to have co-evolved, with one or a few closely related rodent species [2–4]. For example, Hantaan virus (HTNV) is harbored by the striped field mouse (*Apodemus agrarius*); Dobrava-Belgrade virus (DOBV) by the yellow-necked field mouse

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(*Apodemus flavicollis*); Seoul virus (SEOV) by the Brown rat (*Rattus norvegicus*); Thailand virus (THAIV) by the Greater bandicoot rat (*Bandicota indica*); Puumala virus (PUUV) by the bank vole (*Clethrionomys glareolus*); and Prospect Hill virus (PHV) by the meadow vole (*Microtus pennsylvanicus*).

Prompted by the discovery of Sin Nombre virus (SNV) [5] and other sigmodontine rodent-borne hantaviruses causing a rapidly progressive, frequently fatal respiratory illness, known as hantavirus pulmonary syndrome, in the Americas, renewed efforts, aided by sensitive gene amplification techniques, have resulted in the identification of several hantaviruses in Eurasia. Among the more recently detected hantaviruses have been Saaremaa virus (SAAV) in *Apodemus agrarius*, Khabarovsk virus (KHAV) in the reed vole (*Microtus fortis*), Topografov virus (TOPV) in the Brown lemming (*Lemmus sibiricus*), and Tula virus (TULV) in the common vole (*Microtus arvalis*) and the Southern vole (*M. rossiaemeridionalis*) [6].

By virtue of the geographic distribution of *M. arvalis*, TULV is widespread throughout Eurasia. To date, TULV-infected voles have been captured in Central Russia [6], the Slovak [7] and Czech Republics [8,9], Austria [10], Germany [11] and Croatia [12]. Our studies of arvicolid rodents captured in Serbia–Yugoslavia indicate that a less widespread vole species, the European pine vole (*Pitymys subterraneus* or *Microtus subterraneus*) serves as a reservoir of TULV in the Cacak region [13]. We now report the isolation and characterization of TULV from *M. arvalis* in Poland, a country where human hantaviral disease and hantavirus infections of rodents have not been reported.

Materials and Methods

Rodent Trapping and Serology

Sera from murid and arvicolid rodents (70 *Apodemus agrarius*, 16 *Apodemus flavicollis*, 8 *Mus musculus*, 24 *Clethrionomys glareolus*, 9 *Microtus arvalis*, 1 *Pitymys* sp.), captured in Lódz (53 km south and 104 km west of Warsaw) and in Tuszyn (a small town near Lodz), Poland, during June–September 1995, were diluted 1:32 in 0.1 M phosphate-buffered, saline (PBS, pH 7.2) and tested for IgG antibodies to PUUV (strain Hällnäs) by an indirect immunofluorescent antibody (IFA) assay [14]. Lung tissues and spleen tissues from two common voles with antibody to PUUV were then used for virus isolation attempts and genetic studies.

Virus Isolation

Subconfluent monolayers of Vero E6 cells (CRL 1586) (American Type Culture Collection, Rockville, MD), grown in 25 cm^2 flasks, were inoculated with 0.1 ml of 5% (w/v) suspensions of lung and spleen tissues prepared in Dulbecco minimum essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) [15]. Inocula were initially centrifuged at 670 g onto cell monolayers for 2 h at 25°C. Vero E6 cell monolayers were thereafter maintained with DMEM supplemented with 10% FBS and were subcultured at 10- to 14-day intervals. At each passage level, cells were examined for hantavirus antigens by the IFA technique, using convalescent-phase sera from patients with Korean hemorrhagic fever and nephropathia epidemica, as well as sera from experimentally infected rats and mice specific for SEOV and PHV. Cells were also examined for hantaviral RNA by reverse transcription-polymerase chain reaction (RT-PCR) using consensus primers designed from sequences of arvicolid rodent-borne hantaviruses.

Electron Microscopy

TULV-infected Vero E6 cell monolayers were washed with PBS, fixed *in situ* with 2.5% glutaraldehyde for 2 h at 4°C, then scraped and pelleted by centrifugation [16]. Cell pellets were postfixed in 4% osmium tetroxide for 2 h, then dehydrated through graded ethanols and propylene oxide and embedded in Epon 812. Ultrathin sections were examined for hantavirus virions using a Zeiss EM 109 transmission electron microscope at 80 kV.

Experimental Infection in Laboratory Rodents

Small laboratory animals were tested for susceptibility to TULV, using previously described methods [17,18]. Briefly, three groups each of

eight suckling, one-day old Balb C mice, five weaned laboratory-bred striped field mice (*Apodemus agrarius*) and four weaned Mongolian gerbils (*Meriones unguiculatus*) were inoculated by the intracerebral (0.03 ml) and intramuscular (0.5 ml) routes with clarified Vero E6 cell culture fluid from the seventh passage of TULV strain Lodz-2. Mongolian gerbils and striped field mice were selected since they are known to be susceptible to PUUV and HTNV infection, respectively. Signs of clinical illness and death of inoculated animals were monitored for three weeks postinoculation. Sera from inoculated animals were tested for antibodies against TULV by IFA and tissues were examined for TULV RNA by RT-PCR.

Amplification of TULV RNA

Total RNA, extracted using RNAzol (GIBCO/BRL, Gaithersburg, MD) from lung tissues of hantavirus-seropositive *M. arvalis* and from Vero E6 cells infected with TULV isolates (Lodz-1 and Lodz-2), was reverse transcribed using the superscript II RNase H^- reverse transcriptase kit (GIBCO/BRL) [13]. TULV sequences were then amplified by PCR, using previously described oligonucleotide primers for the S genomic segment, 5′-AAGCTACTACTAAAACCGCTGG-3′ + 20, 5′-GCAGATTGGT (T+A) TTC (T+A) ATTGGAC-3′ +391. Primers afforded amplification of the entire nucleocapsid protein-encoding S segment, and a 421-nucleotide region of the Gl and a 948-nucleotide region of the G2 glycoprotein-encoding M segment of TULV. PCR products were cloned using the TA cloning system (Invitrogen Corp., San Diego, CA), and plasmid DNA was purified by the QIAprep-spin Plasmid kit (QIAGEN Inc., Chatsworth, CA). DNA sequencing was performed in both directions from at least three clones of each PCR product, using the dye primer cycle sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, CA) on an automated sequencer (ABI Model 373A).

Amplification of Mitochondrial DNA (mtDNA)

To study the phylogenetic relationships between *M. arvalis* from various geographic regions, the cytochrome b and D-loop regions of mtDNA were amplified by PCR, using previously described universal primers [19,20], which permit amplification of 482- and 1,020-base pair products, respectively. Oligonucleotide primers were used at a final concentration of 0.1 μM in a 100 μl reaction mixture (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP) containing 5 μl of total DNA (25–100 μg/ml) and 2.5 units of AmpliTaq polymerase (Perkin Elmer Co., Norwalk, CT). Reaction mixtures were cycled 40 times: denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C. PCR products were cloned, and DNA was sequenced, as indicated above.

Phylogenetic Analysis

TULV sequences amplified and sequenced from two *M. arvalis* rodents and two Vero E6 cell isolates (strains Lodz-1 and Lodz-2) were aligned and compared with previously published hantavirus sequences [13,21]. The Genbank accession numbers for the TULV Lodz strains are AF063891, AF063892, AF063896, AF063897, and AF063898. Alignment and comparison of the entire nucleocapsid protein-encoding S genomic segment and a 421-nucleotide region of the Gl and a 948-nucleotide region of the G2 glycoprotein-encoding M segment were facilitated by using various programs of the GCG package (Genetics Computer Group Inc., University of Wisconsin, Madison, WI). For phylogenetic analysis, the neighbor-joining (N-J) method, unweighted pair-group method of assortment (UPGMA), Fitch–Margoliash method of PHYLIP (Phylogenic Inference Package, version 3.5c) [22], and maximum parsimony PAUP (Phylogenetic Analysis Using Parsimony, version 4.0) [23] were used. The genetic distances were computed by the DNADIST program using the Kimura two-parameter method [24], and tree structures were inferred using the N-J, UPGMA and Fitch–Margoliash methods with the global rearrangement option.

Results

Epizootiologic Survey

A total of 130 rodents, principally field mice (*Apodemus agrarius* and *Apodemus flavicollis*),

Table 1. Field survey of hantavirus infection in rodents captured in Lodz, Poland, in 1995

Rodent species	No. of rodents captured	No. of seropositive (%)
Apodemus agrarius	70	5 (7.1)
Apodemus flavicollis	16	1 (6.3)
Microtus arvalis	9	3 (33.3)
Pitymys sp.	1	0 (0)
Clethrionomys glareolus	24	3 (12.5)
Mus musculus	8	2 (25)

Sera, diluted 1:32, with IgG antibodies to PUUV, as determined by an indirect immunofluorescent, antibody assay, were considered positive.

were captured in Lodz and Tuszyn, Poland, during June–September 1995. Approximately 7% (6/86) of the *Apodemus* rodents were hantavirus seropositive. Of 34 arvicolid rodents, sera from three *M. arvalis* and three *C. glareolus* contained IgG antibodies to PUUV, as determined by the IFA technique (Table 1).

Virus Isolation

By direct inoculation of Vero E6 cells, we were able to isolate two TULV strains, designated Lodz-1 and Lodz-2, from lung and spleen tissues of two seropositive *M. arvalis*, captured in July 1995. After 14 days, a few hantavirus-positive cells were detected by IFA, and, 20 days later (34 days post-inoculation), approximately 90% of cells exhibited virus-specific fluorescence. By thin-section electron microscopy, strain Lodz-2 closely resembled typical hantavirus virions, measuring approximately 100 nm in diameter, with distinct surface projections.

Experimental TULV Infection

TULV strains Lodz-1 and Lodz-2 failed to produce fatal meningoencephalitis in infant Balb C mice or to infect weanling, laboratory-bred striped field mice. By contrast, weanling Mongolian gerbils developed asymptomatic infection, and IgG antibodies against TULV were detectable in their sera 3 weeks after inoculation. Moreover, virus-specific fluorescence was detected in lung tissues, and TULV RNA was amplified from various tissues of experimentally infected gerbils at 21 days post-inoculation. TULV was serially passaged in weanling Mongolian gerbils by intracerebral or intramuscular inoculation of 10% clarified RT-PCR-positive lung homogenates.

Mitochondrial DNA Sequence Analysis

The European common voles (0117 and 0123), from which the two TULV strains were isolated, were identified as *M. arvalis* by morphological criteria, and their speciation was verified by mtDNA sequence analysis. Specifically, phylogenetic analysis, based on a 403-base pair sequence of the cytochrome b region of mtDNA, showed that *M. arvalis* clustered according to their geographic origin and was distinct from *M. rossiaemeridionalis* and *Pitymys subterraneus* (Fig. 1). Moreover, European common voles from Poland (0117, 0123 and 0124) were evolutionarily distinct from *M. arvalis* captured in Russia and Serbia–Yugoslavia.

S- and M-Segment Sequence Analysis

Sequences of the complete S segment, amplified from lung tissues of a wild-caught vole (Lodz-1) and from virus-infected Vero E6 cells (Lodz-2), and sequences of part of the G1 and G2 glycoprotein-encoding M segment of strains Lodz-1 and Lodz-2 were analyzed. The entire S genomic segment of strain Lodz-1 was 1852 nucleotides in length, with a predicted nucleocapsid protein of 430 amino acids (starting at nucleotide position 43) and a 517-nucleotide 3′-noncoding region (NCR). A second open reading frame (ORF), starting at nucleotide position 83 and encoding 90 amino acids, was identified at the same position as in previously described TULV strains.

The genetic diversity of strains Lodz-1 and Lodz-2 was 0.1% at the nucleotide level, expressed only as a single amino acid difference. The entire coding region of the S genomic segment of the TULV isolates from Poland diverged from other *Microtus* and *Pitymys* rodent-borne TULV strains by about 15–17% at the nucleotide level and 3–4% at the amino acid level, respectively (Table 2). In the hypervariable region of the nucleocapsid protein, between amino acid residues 244 and 269, TULV strain Lodz-1 diverged by 3 or 4 amino acids from other TULV strains. The Moravia and Malacky strains have a deletion at amino acid

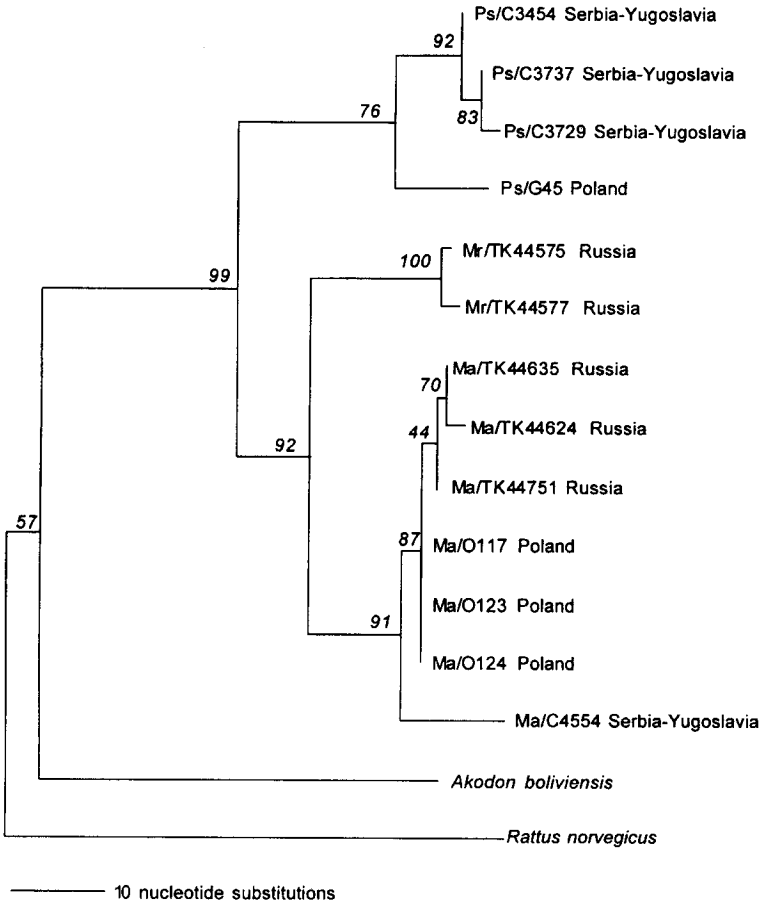

Fig. 1. A phylogenetic tree based on the 403-base pair cytochrome b region of mitochondrial DNA. As depicted, *Microtus arvalis* (Ma) rodents are distinct from *M. rossiaemeridionalis* (Mr) and *pitymys* rodents (ps), and *Microtus* and *pitymys* rodents cluster according to their geographic origin. Ma/0117, Ma/0123, Ma/0124 and Ps/G45 were captured in Poland, Mr/TK44575, Mr/TK44577, Ma/TK44635, Ma/TK44624 and Ma/TK44751 from Ukraine, and Ma/C4554 and Ps/C3454 from Serbia–Yugoslavia.

Table 2. Nucleotide and amino acid sequence homologies of the entire coding region of the S segment and partial G2 glycoprotein-encoding M segment between Tula virus (TULV) strain Lodz-2 and other arvicolid rodent-borne hantaviruses (%)

	S segment		M segment	
Virus strain	1290 nt	430 aa	948 nt	316 aa
TULV Lodz-1	99.9	99.8	100	100
TULV C3454	85.1	97.4	82.3	98.7
TULV M5302	85.2	96.5	83.5	99.4
TULV T23	83.9	96.3	ND	ND
ISLAV MC-47	73.3	84.9	ND	ND
BLLV MO-46	75.5	83.7	ND	ND
KHAV MF-43	ND	ND	75.9	83.5
PHV PH-1	75.3	81.2	75.8	85.8

Abbreviations: TULV, Tula virus; ISLAV, Isla Vista virus; BLLV, Bloodland Lake virus; KHAV, Khabarovsk virus; PHV, Prospect Hill virus; nt, nucleotide; aa, amino acid; ND, not determined because of insufficient sequence data

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                                244                 269
TULV/Lodz-2 (Poland)            CPFLKKQGGPNK---DEEFLVSNDAY
TULV/T23 (Russia)               ........A.G.---E.........
TULV/C3454 (Yugoslavia)         ........A.G.---E.D........
TULV/M5286 (Czech)              ......S.-.S.---E.D........
TULV/Ma32 (Slovakia)            ......S.-.S.---E.D........
TULV/C109 (Croatia)             ......S.-.S.---E.D........
TULV/G20 (Germany)              ......S.-.S.---E.D........
TULV/MG23 (Russia)              .........-ST.---E.........
KHAV/MF-43 (Russia)             ...I.PEVK.GTPAGEA...SR.QL.
ISLAV/MC-47 (USA)               .....SEAR.GQPAEEAAI.S..R..
BLLV/MO-46 (USA)                .....AEPR.GQAVGES...S..RV.
PHV/PH-1 (USA)                  .....AEPR.GQPAGEA...S.IR..
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Fig. 2. Alignment and comparison of deduced amino acid sequences of the hypervariable region of the nucleocapsid-encoding S genomic segment of *Microtus* rodent-borne hantaviruses. Comparison is made between the Tula virus (TULV) strain, isolated from *Microtus arvalis* captured in Lodz, Poland in 1995, and other TULV strains from Russia (T23 and MG23), Serbia–Yugoslavia (C3454), Croatia (C109), Germany (G20), and the Czech (M5286) and Slovak (Ma32) Republics. Sequences of other arvicolid rodent-borne hantaviruses, including Khabarovsk (KHAV), Isla Vista (ISLAV), Bloodland Lake (BLLV) and Prospect Hill (PHV) viruses, are included. TULV strains from Serbia–Yugoslavia (C3454) and from Omsk, Russia (MG23) were from *Pitymys subterraneus* and *Microtus gregalis*, respectively.

position 252. However, TULV strain Lodz-1 had a glycine and TULV strains from Serbia–Yugoslavia and Russia had an alanine at this position (Fig. 2).

Sequence analysis of a 948-nucleotide region of the G2 glycoprotein-encoding M segment revealed that the TULV from Poland differed by more than 16% from TULV of other geographic origins. However, at the amino acid level, the 316-amino acid region of G2 glycoprotein was highly conserved. A 421-nucleotide region (nucleotide position 1227–1647) of the Gl glycoprotein-encoding M segment of strain Lodz-2 diverged from the Moravia strain from the Czech Republic by 19% at the nucleotide level and 7% at the amino acid level, respectively. Gl envelope glycoprotein sequences were more variable than G2 glycoprotein sequences.

Phylogenetic Analysis

Phylogenetic analysis by PAUP, based on the entire coding region of the S segment, indicated three separate, well-supported subclades of TULV strains: those from Poland; those from Russia; and those from Croatia, the Czech Republic, Germany and Serbia–Yugoslavia (Fig. 3). Similarly, a majority-rule consensus phylogenetic tree, based on a 948-nucleotide region of the G2 glycoprotein-encoding M segment, indicated that the TULV hantavirus strains Lodz-1 and Lodz-2 shared a common ancestry with TULV strains from elsewhere in Europe. Both of these topologies were strongly supported by bootstrap analysis of 1000 iterations.

Discussion

Hantaviruses and HFRS are widespread across Scandinavia and Eurasia [25–21]. The outbreaks of HFRS (or Tula fever) in Central Russia in the 1930s occurred coincident with that of nephropathia epidemica in Scandinavia and hemorrhagic nephrosonephritis and Songo fever in Far Eastern Russia and Manchuria, along the Amur River basin [25,26] . Within the European continent itself and outside of Scandinavia, PUUV, DOBV, SAAV and TULV are the principal hantaviruses. Hantavirus-caused human diseases have been most frequently reported in European Russia, France, Germany, Belgium and Serbia–Yugoslavia. Cases have been less commonly diagnosed in the Netherlands, Denmark, Slovenia, Slovakia, Bosnia-Herzegovina, Greece, Estonia, Latvia, Austria, Portugal and the Czech Republic [27].

That human hantavirus disease has not been recognized in Poland is somewhat surprising, given that the murid and arvicolid rodent species known to harbor pathogenic hantaviruses (such as *Apodemus agrarius* and *Clethrionomys glareolus*) are extant in Poland. Not unexpectedly, our limited serosurvey indicated serological evidence of hantavirus infection in these rodent species. Thus, the probability of PUUV and SAAV in Poland is

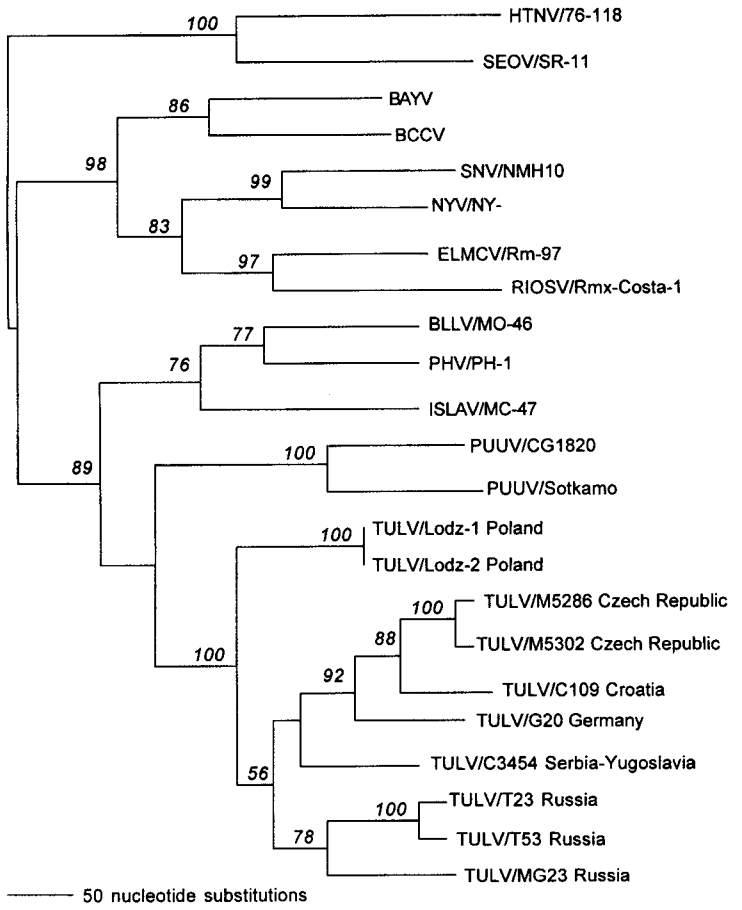

Fig. 3. A majority-rule consensus phylogenetic tree, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire coding region of the S segment of TULV strains. The tree indicates phylogenetic clustering according to the geographic origin of TULV strains from Poland (Lodz-1 and Lodz-2), Serbia–Yugoslavia (C3454), Russia (T23, T53 and MG23), the Czech Republic (M5286 and M5302), Croatia (C109) and Germany (G20). The phylogenetic positions of HTNV, SEOV, El Moro Canyon virus (ELMCV), Rio Segundo virus (RIOSV), SNV, New York virus (NYV), Bayou virus (BAYV), Black Creek Canal virus (BCCV), PUUV, Prospect Hillvirus (PHV), Bloodland Lake virus (BLLV) and Isla Vista virus (ISLAV) are also shown. Branch lengths are proportional to the number of nucleotide substitutions, while vertical distances are for clarity. The numbers at each node are bootstrap probabilities (expressed as percentages), as determined for 1000 iterations by PAUP version 4.0. The GenBank accession numbers for the S segment of TULV strains Lodz-1 and Lodz-2 are AF063892 and AF063897, respectively.

very high. For the purposes of this report, however, we have focused on the isolation and characterization of TULV.

Originally identified in the common vole (*Microtus arvalis*) and the Southern vole (*M. rossiaemeridionalis*) captured in the Tula region of Central Russia [6], TULV has since been identified in *M. arvalis* captured in the Slovak [7] and Czech Republics [8,9], Austria [10] and Germany [11], as well as in *Pitymys subterraneus* captured in Serbia–Yugoslavia [13]. Recently, TULV has been detected more commonly in field voles (*Microtus agrestis*) than in European common voles (*M. arvalis*) captured in training areas utilized by US Forces in Croatia [12]. The demonstration of TULV-infected *M. arvalis* in Poland, by virus isolation (directly into Vero E6 cells) and by gene amplification, serves as clear evidence that TULV is present in Poland.

Although an etiologic association between TULV infection and human disease has not been made [8,11,28], the isolation of TULV and the

existence of hantavirus-seropositive *A. agrarius* and *C. glareolus* in Poland should alert Polish clinicians to be vigilant for hantavirus disease in humans. The detection of antibodies against hantaviruses among residents of neighboring Hungary, and the demonstration of PUUV and DOBV sequences in rodents captured in Hungary [29], should provide additional incentive to more thoroughly investigate the ecology of hantaviruses and the human disease burden from hantavirus infection in Poland. Although a study of chronic dialysis and suspected-leptospirosis patients and at-risk populations (such as animal caretakers and farmers) in Poland showed no convincing serological evidence of hantavirus infection [30], intensified efforts are nevertheless warranted.

Like other arvicolid rodent-borne hantaviruses (such as PUUV and PHV), the new TULV isolates from Poland did not infect infant mice or weaned, laboratory-bred striped field mice (*A. agrarius*). Weanling Mongolian gerbils, on the other hand, were highly susceptible to experimental TULV infection. Moreover, these data confirm that Mongolian gerbils serve as a useful laboratory animal host for propagating hantaviruses [18].

The Lodz strain of TULV, reported here, shared a common ancestry with other TULV strains. The degree of genetic variability in the S genomic segment between TULV strains from Serbia–Yugoslavia, Central Russia and the Czech Republic was similar to that for PUUV strains from Finland and Russia [31,32]. As for *Clethrionomys*-borne strains of PUUV, sequence and phylogenetic analyses indicated geographic-specific phylogenetic clustering of *Microtus*-borne TULV strains. This observation can readily be attributed to a founder effect and to the relatively limited geographic range of arvicolid rodents.

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