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Hokkaido Genotype of Puumala Virus in the Grey Red-backed Vole (*Myodes rufocanus*) and Northern Red-backed Vole (*Myodes rutilus*) in Siberia

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Abstract

Three species of *Myodes* voles known to harbor hantaviruses include the bank vole (*Myodes glareolus*), which serves as the reservoir host of Puumala virus (PUUV), the prototype arvicolid rodent-borne hantavirus causing hemorrhagic fever with renal syndrome (HFRS) in Europe, and the grey red-backed vole (*M. rufocanus*) and royal vole (*M. regulus*) which carry two PUUV-like hantaviruses, designated Hokkaido virus (HOKV) and Muju virus (MUJV), respectively. To ascertain the hantavirus harbored by the northern red-backed vole (*M. rutilus*), we initially screened sera from 233 *M. rutilus,* as well as from 90 *M. rufocanus* and 110 *M. glareolus*, captured in Western and Eastern Siberia during June 2007 to October 2009, for anti-hantaviral antibodies. Thereafter, lung tissues from 44 seropositive voles were analyzed for hantavirus RNA by reverse transcription-polymerase chain reaction. Partial L-, M- and S-segment sequences, detected in *M. rutilus* and *M. rufocanus*, were closely related to HOKV, differing from previously published L-, M- and S-segment sequences of HOKV by 17.8–20.2%, 15.9–23.4% and 15.0–17.0% at the nucleotide level and 2.6–7.9%, 1.3–6.3% and 1.2–4.0% at the amino acid level, respectively. Alignment and comparison of hantavirus sequences from *M. glareolus* trapped in Tiumen Oblast showed very high sequence similarity to the Omsk lineage of PUUV. Phylogenetic analysis, using neighbor-joining, maximal likelihood and Bayesian methods, showed that HOKV strains shared a common ancestry with PUUV and exhibited geographic-specific clustering. This

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report provides the first molecular evidence that both *M. rutilus* and *M. rufocanus* harbor HOKV, which might represent a genetic variant of PUUV.

Keywords

hantavirus; Puumala virus; *Myodes* voles; Siberia

1. Introduction

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) possess a negative-sense, single-stranded, tripartite RNA genome, comprising L, M and S segments, which encode an RNA-dependent RNA polymerase (RdRp), envelope glycoproteins (Gn and Gc) and nucleocapsid (N protein), respectively (Plyusnin et al., 1996). Several hantaviruses, harbored by rodents (order Rodentia) belonging to the Muridae and Cricetidae families, cause hemorrhagic fever with renal syndrome (HFRS) (Jonsson et al., 2010). Hantaan virus (HTNV), Dobrava-Belgrade virus (DOBV) and Seoul virus (SEOV) are the principal pathogenic murid rodent-borne hantaviruses, while Puumala virus (PUUV), carried by the bank vole (*Myodes glareolus*), is the prototype HFRS-causing arvicolid rodent-borne hantavirus.

M. glareolus is one of the most abundant and widely distributed small mammal species in Europe, occurring in most areas except near the coast of the Mediterranean Basin and in the far northern territories (Olsson et al., 2010). In the Asian part of the continent, its range extends to the Sayan Mountains and northern Kazakhstan (Shenbrot and Krasnov, 2005). Following the latest glacial period, *M. glareolus* recolonized the continent and formed at least eight distinct lineages of co-evolved PUUV (Sironen et al., 2001). Phylogenetic studies suggest that the Omsk lineage of PUUV in western Siberia has a common evolutionary origin with that of the Finnish lineage (Dekonenko et al., 2003).

Three other species of *Myodes* voles serve as reservoir hosts of hantaviruses: the grey red-backed vole (*M. rufocanus*), northern red-backed vole (*M. rutilus*) and royal vole (*M. regulus*). The former two species have overlapping geographic ranges with *M. glareolus* in Europe and Asia (Shenbrot and Krasnov, 2005). In particular, *M. rufocanus* is distributed in the northern Paleoarctic, extending from northern Fennoscandia through northern Russia to Kamchatka, northeastern and northern Korea, Mongolia, China, Sakhalin (Russia) and Hokkaido (Japan), whereas *M. rutilus* is a Holarctic species found throughout northern Europe, Asia, Alaska and Canada. By contrast, *M. regulus* is confined to mountainous regions at elevations above 500 m in the Korean Peninsula (Kaneko, 1990).

Hantaviral antigens were initially detected in *M. rufocanus* in Far East and Siberian Russia (Kosoy et al., 1997; Tkachenko et al., 1983, 1987). Subsequently, genetic evidence of a PUUV-like hantavirus, designated Hokkaido virus (HOKV), was demonstrated in *M. rufocanus* trapped in Hokkaido, Japan (Kariwa et al., 1995, 1999; Sanada et al., 2012), the Far East region and Buryatia Republic of Russia (Plyusnina et al., 2008; Yashina et al., 2004, 2013) and Jilin province, China (Zhang et al., 2007). Another PUUV-like virus, named Muju virus (MUJV), was detected in *M. regulus*, trapped at multiple sites in the Republic of Korea

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(Lee et al., 2014; Song et al., 2007). Both HOKV and MUJV share a common ancestry with PUUV.

Although hantaviral antigens or antibodies have been reported in *M. rutilus*, captured in Alaska (Lee et al., 1982) and in European, Siberian and Far East Russia (Kosoy et al., 1997; Tkachenko et al., 1987; Yashina et al., 2012), the hantavirus harbored by *M. rutilus* has not been characterized. In the present study, genetic analysis of hantaviruses in *M. rutilus, M. rufocanus* and *M. glareolus*, sharing the same ecological habitats in the Western Siberia, indicates that both *M. rutilus* and *M. rufocanus* serve as reservoir hosts of HOKV. Based on whole genome sequence analysis of MUJV (Lee et al., 2014) and findings from this investigation, HOKV and MUJV appear to represent genetic variants, or genotypes, of PUUV, rather than distinct hantavirus species. We, therefore, tentatively propose that HOKV and MUJV be called the Hokkaido and Muju genotypes of PUUV.

2. Materials and methods

2.1. Rodent trapping and screening

During June 2007 to October 2009, rodents were captured in 12 forest localities of seven administrative regions of Western and Eastern Siberia (Altai Republic, Altai and Krasnoyarsk Krais, and Tiumen, Omsk, Kemerovo and Tomsk Oblasts), where two or three *Myodes* species were sympatric (Table 1 and Fig. 1). Captured *Myodes* voles were identified according to a complex of morphologic criteria, including configuration of the prisms and triangles of the occlusal surface of the third upper (M^3) and first lower (M_1) molars, body size, length and hairiness of tail, and fur coloration (Gromov and Erbaeva, 1995; Gromov and Polyakov, 1977). Sera and lung tissues were stored in liquid nitrogen for subsequent analysis for anti-hantavirus antibodies by the indirect immunofluorescent antibody test (IFA) using Vero E6 cells infected with HTNV (76–118), PUUV (CG1820) and SEOV (SR-11) as antigens (Dzagurova et al., 1995), and for hantavirus RNA by reverse transcription-polymerase chain reaction (RT-PCR) (Yashina et al., 2004, 2013). Although hantaviral antigens have been detected before antibody responses in *M. glareolus* experimentally infected with PUUV (Apekina et al., 2014), we were unable to test all tissues from wild-trapped *Myodes* voles by RT-PCR, due to budgetary constraints, and instead were able to test tissues only from IFA-seropositive voles.

2.2. Ethics statement

All trapping and handling of rodents and processing of their tissues were performed according to well-established protocols (Mills et al., 1995), approved by the Institutional Animal Care and Use Committee of the State Research Center of Virology and Biotechnology “Vector”.

2.3. RT-PCR, mtDNA and DNA sequencing

Total RNA was extracted from lung tissue of anti-hantaviral antibody-positive rodents with the RNeasy MiniKit (Qiagen, Hilden, Germany), and cDNA was synthesized using Expand reverse transcriptase (Roche) and universal primer 5′-TAGTAGTAGACTCC-3′. Four sets of nested primers were used to amplify selected regions of the large (L: positions 180–522),

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medium (M: 2764–3004) and small (S: 43–1251) segments (nucleotide position numbers are those for PUUV strain CG1820: GenBank M63194, M29979, M32750). Sequences of primers are shown in Table 2. To confirm the taxonomic identity of hantavirus RNA-positive voles, genomic DNA was extracted from frozen lung tissue using the QIAamp DNA Mini Kit (Qiagen), and the partial 426-nucleotide region of the cytochrome *b* gene of mitochondrial DNA (mtDNA) was amplified by PCR using previously described universal primers: +14115 (5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′); and −14532 (5′-GCAGCCCCTCAGAATGATATTTGTCCAC-3′) (Smith & Patton, 1991). Amplicons were gel-purified with QIAquick Gel Extraction kit (Qiagen) and directly sequenced using ABI Prizm BigDye Terminator kit (PE Applied Biosystem) and an automatic ABI Prism 310 genetic analyzer.

2.4. Genetic and phylogenetic analyses

Pair-wise alignment and comparison of newfound hantavirus nucleotide sequences were performed using ClustalW (Thompson et al., 1994). The distance-based neighbor-joining method, supported by MEGA 5.1 (Tamura et al., 2011), was used to construct phylogenetic trees, which were evaluated by bootstrap analysis of 1,000 neighbor-joining iterations. In addition, unrooted phylogenetic trees were generated by maximum likelihood and Bayesian methods, implemented in PAUP* (Phylogenetic Analysis Using Parsimony, 4.0b10) (Swofford, 2003), RAxML Blackbox webserver (Stamatakis et al., 2008) and MrBayes 3.1 (Ronquist and Huelsenbeck, 2003), under the best-fit GTR+I+Γ model of evolution selected by hierarchical likelihood-ratio test in MrModeltest v2.3 (Posada and Crandall, 1998) and jModelTest version 0.1 (Posada, 2008). Two replicate Bayesian Metropolis–Hastings Markov Chain Monte Carlo runs, each comprising six chains of 10 million generations sampled every 100 generations with a burn-in of 25,000 (25%), resulted in 150,000 trees overall. Each genomic segment (S, M and L) was treated separately in phylogenetic analyses. The posterior node probabilities were based on 2 million generations and estimated sample sizes over 100 (implemented in MrBayes).

3. Results

M. rutilus was the dominant *Myodes* species captured at six sites, *M. rufocanus* at three and *M. glareolus* at two, with equal numbers of *M. rutilus* and *M. glareolus* at one site (Omsk Oblast) (Table 1). Sera, diluted 1:10, from 433 voles (233 *M. rutilus,* 90 *M. rufocanus* and 110 *M. glareolus*) were initially screened by IFA for the presence of anti-hantaviral antibodies. Individuals of one, two or three *Myodes* species in 11 of the 12 trap sites were anti-hantavirus antibody positive (Table 1).

Of the 44 (10.2%) seropositive voles, hantavirus RNA was detected in two of 17 *M. rutilus*, two of 19 *M. rufocanus* and three of eight *M. glareolus*, which were taxonomically verified by mitochondrial DNA analysis. Sequence analysis, based on a 426-nucleotide cytochrome *b* region of mtDNA, showed that intraspecies differences in *Myodes* voles (*M. glareolus*, *M. rufocanus* and *M. rutilus*) varied from 0 to 2.3%, while interspecies differences were from 6.8–9.6%. In all studied cases, cytochrome *b* typing matched morphological specification of

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hantavirus RNA-positive *Myodes* voles. The GenBank accession numbers for the cytochrome b sequences are provided in Table 3.

Hantavirus RNA-positive voles were captured in three localities of the Altai region: near Teletskoye Lake (Artybash village, Altai Republic), Pokrovka and Solton villages (Altai Krai) and in one locality of Tiumen Oblast near Kuchuk Lake (Table 1 and Fig. 1). In one of these sites (Teletskoye Lake), hantavirus RNA was detected in two of three hantavirus-infected *Myodes* species (*M. rutilus* and *M. rufocanus*), and in each of three other sites hantavirus sequences from a single host species were identified.

Hantavirus RNA was detected in lung tissues from seven of 44 anti-hantaviral antibody-positive *Myodes* voles. Of these, six voles had anti-hantavirus antibody titers ≥1:80 (Table 4). Six of 17 voles with antibody titers ≥1:80 were hantavirus RNA positive, compared to one of 27 voles with anti-hantaviral antibody titers ≤1:40. At titers of ≥1:320, four of five voles were hantavirus RNA positive (Table 4).

Analysis of partial L- (position 180–522), M- (position 2764–3004) and S- (position 43–1251) segment sequences from *M. rutilus* and *M. rufocanus* showed that both species harbored a hantavirus which was most closely related to HOKV. HOKV was detected in *M. rutilus* in Solton, in *M. rufocanus* in Pokrovka and in both *M. rutilus* and *M. rufocanus* in Teletskoye Lake (Table 1). In both Solton and Teletskoye Lake, *M. rutilus* was the dominant species, accounting for 53.8% and 86.0% of all captured *Myodes* voles, respectively.

The nucleotide sequence similarity among the newly identified HOKV strains Teletskoye854/Mrt, Teletskoye937/Mrf, Solton35/Mrt and Pokrovka674/Mrf were 83.6–97.7%, 80.5–98.8% and 84.5–97.6% for the L-, M- and S-segments, respectively. And the deduced amino acid sequence similarity was 99.1–100% for the RdRp, 98.8–100% for the Gc glycoprotein and 97.3–99.3% for the N protein. Minimal nucleotide sequence divergence (<3%) was shown for HOKV strains, Teletskoye854/Mrt and Teletskoye937/Mrf detected in *M. rutilus* and *M. rufocanus*, respectively, from one locality. Compared with previously published HOKV L-, M- and S-segment sequences from *M. rufocanus*, 79.8–82.2%, 76.6–84.1% and 83.0–85.0% similarity was found at the nucleotide level and 92.1–97.4%, 93.7–98.7% and 96.0–98.8% at the amino acid level. The amino acid sequences of the RdRp, Gc and N proteins of the Siberian HOKV strains differed from that of PUUV strains by 6.1–9.7%, 5.0–6.3% and 3.5–4.5%, and from that of MUJV strains by 9.7–10.5%, 5.0–6.3% and 4.2–6.0%.

Hantavirus RNA, detected in three of four seropositive *M. glareolus* in Tiumen Oblast (Kuchuk170/Mg, Kuchuk197/Mg, Kuchuk246/Mg), showed the highest degree of sequence similarity to the Omsk lineage of PUUV, previously described in *M. glareolus* captured in a neighboring administrative region of Siberia (Omsk Oblast): 93.3–96.7% and 97.5% for M and 96.6–96.9% and 98.5–99.2% for S segment at the nucleotide and amino acid levels, respectively. Compared with other PUUV strains, Kuchuk170/Mg, Kuchuk197/Mg and Kuchuk246/Mg differed by 14.5–19.6% at the nucleotide level and 1.8–6.1% at the amino acid level for the L segment, and by 14.1–17.4% and 3.7–8.7% for the M segment, and by 11.2–17.4% and 1.7–4.7% for the S segment, respectively. The partial amino acid sequences

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of the Siberian HOKV strains differed from that of the Siberian PUUV strains by 6.1–7.9% for RdRp, 6.2–7.5% for Gc and 3.7–5.0% for N protein.

Phylogenetic trees based on partial S-, M- and L-segment sequences, using neighbor-joining, maximum-likelihood and Bayesian methods, indicated that HOKV strains were placed within the well-supported clade formed by three hantaviruses associated with *Myodes* voles: PUUV, HOKV and MUJV (Fig. 2). HOKV strains formed five genetic lineages, represented respectively by previously described strains from Japan (Kamiiso-8Cr-95, Tobetsu60Cr, Kiritappu126S, Nakagawa49S, Ishikari9S) and Russia (Sakhalin99S), Republic Buryatia (Mukhorshibir767, Tunka227), Russia-China (Fusong and Khekhtsir3S) and two new lineages from Russia: Siberia 1 (Solton35/Mrt, Teletskoye854/Mrt, Olhon109/Mrf) and Siberia 2 (Pokrovka674/Mrf). The N protein sequence of the newfound HOKV strains contained specific amino acid signatures of HOKV (Val/Ile68, Val/Ala79 and Ile262) and residues common for all HOKV strains and those also shared by MUJV, Khabarovsk virus (KBRV) and Topografov virus (TOPV) (Arg26, Pro283). Specific amino acid signatures for the Siberia 1 lineage were Met127, Asp302 and Asp307, and for the Siberia 2 lineage Gln237.

The Siberia 1 lineage of HOKV included three new strains from the Altai region (Solton35, Teletskoye854, Teletskoye937) and the previously described strain Olhon109 from *M. rufocanus* captured in Olhon district of Irkutsk Oblast (Yashina et al., 2013), located more then 1,750 km to the east, near Baikal Lake. The Siberia 2 lineage was found in *M. rufocanus* captured at one site near Pokrovka village (Altai Krai), located approximately 260 km southwest from the two other sites, Teletskoye Lake and Solton. The newly identified PUUV strain Kuchuk170 from *M. glareolus* was placed within the Omsk lineage of PUUV, according to geography.

4. Discussion

The principal objectives of this study were to determine the molecular phylogeny of hantaviruses harbored by three species of *Myodes* voles inhabiting the same natural foci in Western and Eastern Siberia and to fill a long-standing gap in knowledge about the hantavirus harbored by *M. rutilus*. This report provides the first genetic evidence that HOKV is harbored by both *M. rufocanus* and *M. rutilus* in Siberian Russia. That HOKV was found irrespective of whether the dominant *Myodes* species was *M. rutilus* (Teletskoye Lake and Solton) or *M. rufocanus* (Pokrovka) suggests that both species are natural reservoirs of HOKV.

Host-specificity studies of experimental PUUV infection in wild-trapped and colonized cricetid rodents (Klingström et al., 2002) are compatible with our finding of HOKV RNA in lung tissues of two species of anti-hantaviral antibody-positive, wild-trapped voles, *M. rufocanus* and *M. rutilus*. Because *M. rutilus* was the dominant *Myodes* species over *M. rufocanus* in Solton and Teletskoye Lake, spillover infection of HOKV from the latter to the former is unlikely.

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Overall, hantavirus RNA was detected in only 15.9% (7/44) of the anti-hantaviral antibody-positive *Myodes* voles. The discordance between the prevalence of anti-hantaviral antibody and hantavirus RNA was most striking in *M. rufocanus* (2/19, or 10.5%) and *M. rutilus* (2/17 or 11.8%), compared to *M. glareolus* (3/8, or 37.5%) (Tables 1 and 4). However, this discrepancy may have been artificially accentuated by including 12 voles (6 *M. rutilus*, 4 *M. rufocanus* and 2 *M. glareolus*) with anti-hantaviral antibody titers of ≤1:20. That is, hantavirus RNA was more commonly detected in voles with antibody titers ≥1:80 (6/17, or 35.3%) or ≥160 (5/8, or 62.5%), which is consistent with experimental PUUV infection studies, which showed that 48% of voles with high levels of antibodies correlated with PUUV antigen in lung tissues (Apekina et al., 2014).

A possible reason for the low success rate of detecting hantavirus RNA in seropositive voles include the insufficiently sensitive RT-PCR assay; that is, although the L-segment oligonucleotide primers were broadly cross reactive and have been successfully employed for gene amplification of KBRV in *Microtus maximowiczii*, Vladivostok virus (VLAV) in *Microtus fortis* and Tula virus (TULV) in *Microtus arvalis*, the most reasonable explanation for the low RT-PCR positivity in lung tissues among antibody-positive *M. rufocanus* and *M. rutilus* was the lower viral load, which was beyond the limits of detection. Few studies have compared two of these hantaviral markers in *M. rufocanus*. For example, only 5 of 8 seropositive wild *Microtus* and *Myodes* voles were RT-PCR positive (Plyusnina et al., 2008).

Another possible reason for the discrepancy is that HOKV infection may be less robust and persistent in *M. rutilus* and *M. rufocanus*, resulting in lower viral copy numbers, compared to PUUV infection in *M. glareolus*. Thus, while 75% (3/4) of seropositive *M. glareolus* from Kuchuk was PUUV RNA positive, only 12.5% (1/8) of seropositive *M. rufocanus* and 16.7% (1/6) *M. rutilus* were HOKV RNA positive in Pokrovka and Teletskoye Lake, respectively.

By contrast, passively acquired maternal antibodies cannot explain the anti-hantaviral antibody prevalence, because all of the captured voles were subadults and adults. Equally unlikely is the occurrence of spillover and the failure to capture another arvicolid rodent species that represents the reservoir host of HOKV. Only two or three species of *Myodes* voles, which are potential reservoirs of PUUV and PUU-like virus, inhabit the studied territory and all of these species were trapped and analyzed. No other *Myodes* species are described in Siberia.

We were unable to detect PUUV in one seropositive *M. glareolus* captured in Teletskoye Lake, where HOKV was found in seropositive *M. rutilus* and *M. rufocanus*. Similarly, in the trapping site of Kuchuk in Tiumen Oblast, where PUUV was detected in the dominant *M. glareolus*, we failed to find HOKV in one seropositive *M. rutilus*. These results are consistent with that of previous studies, indicating hantavirus antigen in *M. glareolus* and *M. rutilus* within the same natural focus, where *M. glareolus* was the dominant species (Myasnikov et al., 1992).

Geographic-specific clustering within distinct genetic lineages has been recognized for arvicolid rodent-borne hantaviruses, such as PUUV (Plyusnin et al., 1995; Sironen et al., 2001), MUJV (Lee et al., 2014; Song et al., 2007) and TULV (Song et al., 2004; Tkachenko

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et al., 2015). Available data on PUUV support the hypothesis that the phylogeography of different lineages was formed by separate post-glacial migrations of *M. glareolus* (Asikainen et al., 2000). We suggest a similar basis for the distribution pattern of distinct lineages of HOKV. Previously, it was shown that the vast territory of European and Asian Russia, including the southern regions of Siberia, was colonized by the same haplogroup of *M. rufocanus* (Abramson et al., 2012). In accordance with these data, we found genetically closely related HOKV strains of Siberia 1 lineage in the geographically distant Altai region and Baikal Lake area (Teletskoye854/Mrt, Teletskoye937/Mrf, Solton35/Mrt and Olhon109). Geographically close but genetically distant Siberia 2 and Siberia 1 lineages were found in the mountainous area of the Altai region, suggesting that the two lineages are associated with different colonization events of *M. rufocanus*, which had long periods of isolated evolution.

Discovery of multiple newfound hantaviruses raises critical questions about their taxonomic relationships (Lee et al., 2014; Maes et al., 2009) and evolutionary origins (Yanagihara et al., 2014). Our data and results from other studies on the genetic diversity of PUUV, MUJV and HOKV (Lee et al., 2014; Plyusnina et al., 2008; Sironen and Plyusnin, 2011; Zhang et al., 2007) indicate that the current criterion of at least a 7% amino acid sequence difference in both the complete N protein and GnGc, promulgated by the International Committee on the Taxonomy of Viruses (ICTV) (Plyusnin et al., 2012), is not fulfilled to warrant separate hantavirus species designation for PUUV, HOKV and MUJV. In this study, differences between the N protein sequences (based on nearly the complete N protein, 402 of 433 amino acids) of Siberian HOKV and PUUV strains were 4.8–6.0%. Recently, based on a thorough genetic analysis of DOBV strains, a consortium of European hantavirus experts proposed four genotypes: namely Dobrava, Saaremaa, Kurkino and Sochi (Klempa et al., 2013). Similarly, analysis of publicly available sequences of hantaviruses harbored by *Myodes* voles supports the concept of three PUUV genotypes: Puumala, Hokkaido and Muju.

Also, the ICTV criterion requiring a unique ecological niche of the reservoir host is invalid for HOKV. *M. rufocanus* and *M. rutilus*, which belong to the same family (Cricetidae), subfamily (Arvicolinae) and genus (*Myodes*) and which inhabit the same localities in coniferous and broad-leaved forests of Siberian and Far East Russia, harbor HOKV. Thus, this criterion might need to be revised to simply requiring that reservoir hosts belong to the same genus.

Although approximately 7% of the human population in the Omsk region and 9% in the Tiumen region have anti-hantaviral antibodies (Myasnikov et al., 1987), only low numbers of HFRS cases have been registered in Western Siberia. Serological studies in the Altai Republic and Altai Krai have also revealed anti-hantaviral antibody prevalence of approximately 1–2% (Malkin et al., 1996). It is unclear if the rarity of HFRS in Siberia is due to under-reporting or to the lower pathogenicity of HOKV. Future in-depth studies are warranted to clarify if HOKV and MUJV, like PUUV, cause HFRS.

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Highlights

- Three species of *Myodes* voles occupy the same ecological niche in Siberia
- *Myodes glareolus* harbors Puumala virus in Siberia
- *Myodes rufocanus* and *Myodes rutilus* serve as the reservoir hosts of the Hokkaido genotype of Puumala virus in Siberia

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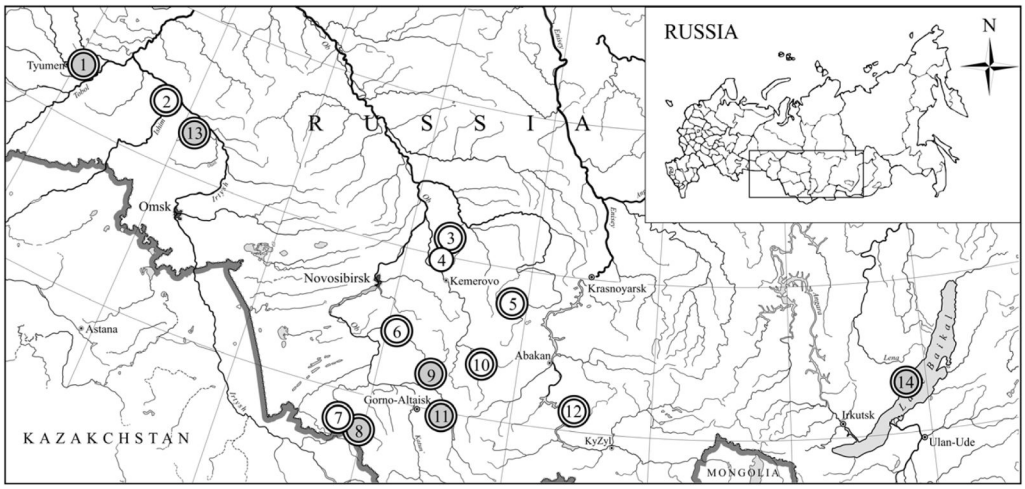

Fig. 1.

Map of Russia, showing the locations of the rodent trapping sites: (1) Kuchuk, (2) Ust-Ishim, (3) Sukhorechie, (4) Balakhnino, (5) Parnaya, (6) Khmelevka, (7) Kolyvan, (8) Pokrovka, (9) Solton, (10) Kuzedeyevo, (11) Teletskoye Lake, (12) and Srednyaya Shush, as well as (13) Omsk region (Dekonenko et. al., 2003) and (14) Olhon district (Yashina et al., 2013). Sites where anti-hantavirus antibody-positive and hantavirus RNA-positive voles were captured are labeled with double and filled circles, respectively

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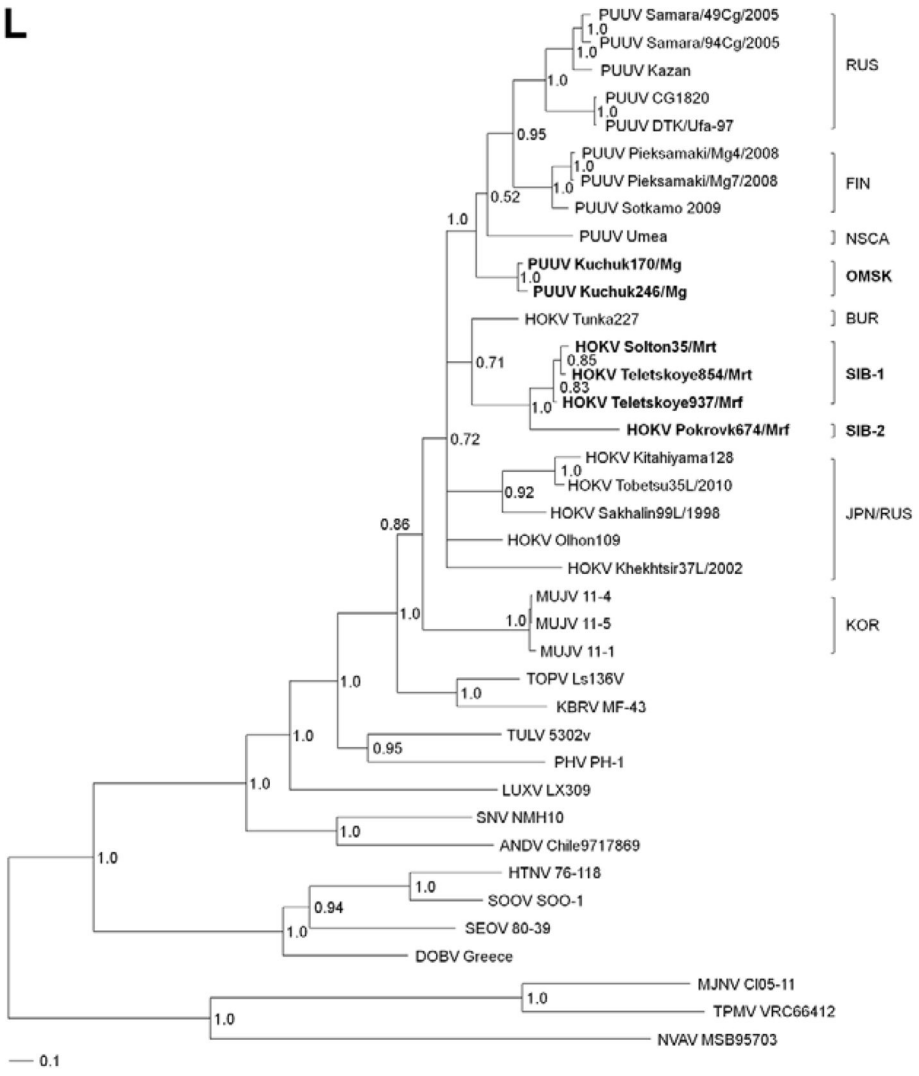

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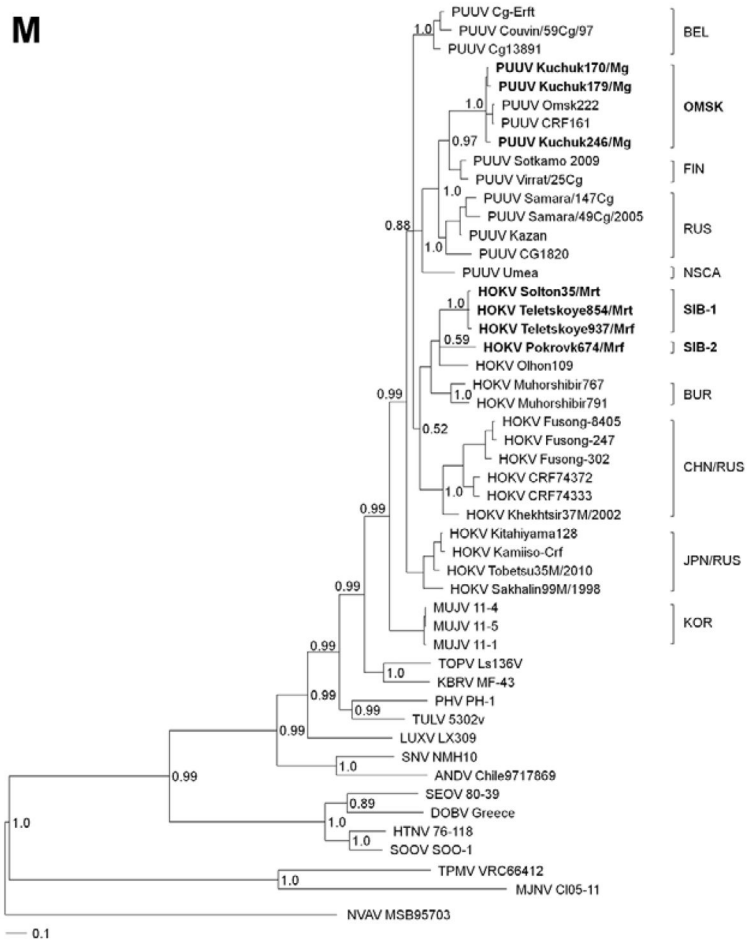

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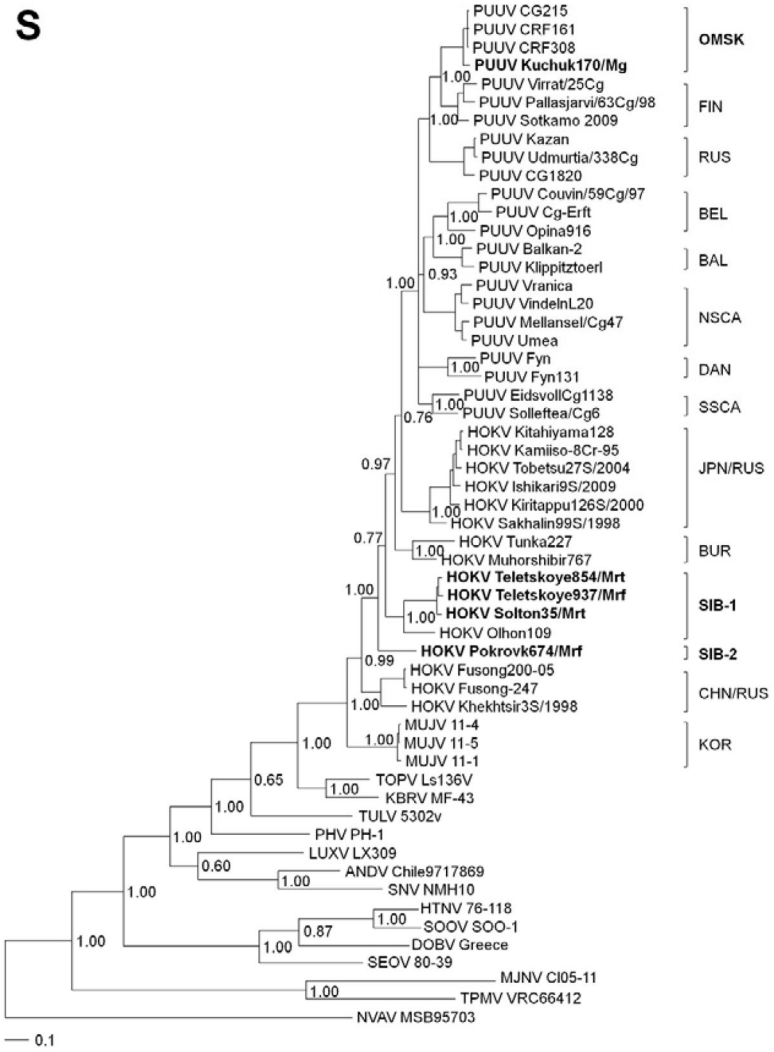

Fig. 2.

Phylogenetic trees, generated by the maximum-likelihood and Bayesian methods, using the GTR+I+Γ model of evolution as estimated from the data, were based on the alignment of the coding regions of the (S) partial S segment (position 43–1251), (M) partial M segment (position 2764–3004) and (L) partial L segment (position 180–522) of *Myodes* vole-borne hantaviruses and other representative hantaviruses. Because the unrooted phylogenetic trees using these methods were very similar, the trees generated by MrBayes were displayed. The phylogenetic positions of the newfound *Myodes* vole-borne hantaviruses from Siberia are shown in relationship to PUUV CG215 (AF367066); PUUV CRF161 (AF367069, AF367061); PUUV CRF308 (AF367070); PUUV Virrat/25Cg (Z69985, Z70201); PUUV Pallasjarvi/63Cg/98 (AJ314597); PUUV Sotkamo (NC_005224, NC_005223, NC_005225); PUUV Pieksamaki/Mg4/2008 (JN831946); PUUV Pieksamaki/Mg7/2008 (JN831945); PUUV Kazan (Z84204, Z84205, EF405801); PUUV Udmurtia/338Cg (Z30708); PUUV CG1820 (M32750, M29979, M63194); PUUV Samara/147Cg (AB433855); PUUV Samara/49Cg/2005 (AB433850, AB574183); PUUV Samara/94Cg/2005 (AB574184); PUUV DTK/

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Ufa-97 (AB297667); PUUV Couvin/59Cg/97 (AJ277034, AJ277040); PUUV Cg-Erft (AJ238779, AJ238778); PUUV Omsk222 (AF442616); PUUV Opina916 (AF294652); PUUV Cg13891 (U22418); PUUV Balkan-2 (AJ314601); PUUV Klippitztoerl (AJ888751); PUUV Vranica (U14137); PUUV Vindeln/L20 (Z48586); PUUV Mellansel/Cg47 (AJ223374); PUUV Umea (AY526219, AY526218, AY526217); PUUV Fyn (AJ238791); PUUV Fyn131 (AJ278092); PUUV Eidsvoll/Cg1138 (AJ223369); PUUV Solleftea/Cg6 (AJ223377); HOKV Kitahiyama128 (AB675463, AB676848, AB712372); HOKV Kamiiso-8Cr-95 (AB010730); HOKV Kamiiso-Crf (AB011631); HOKV Tobetsu27S/2004 (AB675465); HOKV Tobetsu35/2010 (AB675451, AB675452); HOKV Ishikari9S/2009 (AB675469); HOKV Kiritappu126S/2000 (AB675474); HOKV Sakhalin99/1998 (AB675453, AB675454, AB675455); HOKV Tunka227 (KP325675, KR072694); HOKV Muhorshibir767 (AM930972, AM930975); HOKV Muhorshibir791 (AM930976); HOKV Olhon109 (KP325674, KM245956, KM245961); HOKV Fusong 200-05 (EF211820); HOKV Fusong-247 (EF442087, EF442094); HOKV Fusong-302 (EF442095); HOKV Fusong8405 (EF422372); HOKV Khekhtsir3S/1998 (AB677476); HOKV Khekhtsir37/2002 (AB677484, AB677488); HOKV CRF74372 (AY491383); HOKV CRF74333 (AY491381); MUJV 11-1 (JX028273, JX028272, JX028271); MUJV 11-4 (JX046484, JX046483, JX046482); MUJV 11-5 (JX046487, JX046486, JX046485); TOPV Ls136v (AJ011646, AJ011647, AJ011649); KBRV MF-43 (U35255, AJ011648, AJ011650); TULV M5302v (NC_005227, NC_005228, NC_005226); PHV PH-1 (Z49098, X55129, EF646763); and LUXV LX309 (HM756286, HM756287, HQ404253). Other rodent-borne hantaviruses included ANDV Chile9717869 (NC_003466, NC_003467, NC_003468); SNV NMH10 (NC_005216, NC_005215, NC_005217); HTNV 76–118 (NC_005218, Y00386, NC_005222); SOOV SOO-1 (AY675349, AY675353, DQ562292); DOBV Greece (NC_005233, NC_005234, NC_005235); and SEOV 80-39 (NC_005236, NC_005237, NC_005238). Shrew- and mole-borne hantaviruses included MJNV Cl05–11 (EF641804, EF641798, EF641806); TPMV VRC66412 (AY526097, EU001329, EU001330); and NVAV MSB95703 (FJ539168, HQ840957, FJ593498). The corresponding geographic areas are indicated as BAL (Balkan), BEL (Belgium), BUR (Buryatia), CHN/RUS (China/Russia), DAN (Denmark), FIN (Finland), JPN/RUS (Japan/Russia), KOR (Korea), NSCA (Northern Scandinavia), OMSK (Omsk), RUS (Russia), SIB-1 (Siberia), SIB-2 (Siberia), and SSCA (Southern Scandinavia). The numbers at each node are posterior node probabilities based on 150,000 trees. The scale bar indicates nucleotide substitutions per site. The GenBank accession numbers for the newfound hantaviral sequences from Siberia are listed in Table 3.

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Table 1

Prevalence of hantavirus infection, as determined by serology and RT-PCR, in *Myodes* voles captured in Western and Eastern Siberia, 2007–2009

Administrative Region	Trap Locality	Trap Year	*Myodes* Species	Antibody Prevalence	Hantavirus RNA
Altai Krai	Khmelevka	2008	*M. rutilus*	0/13	NT
			M. rufocanus	0/1	NT
			M. glareolus	1/7	0/1
	Kolyvan	2008	*M. rutilus*	1/11	0/1
			M. rufocanus	0/1	NT
	Pokrovka	2008	*M. rutilus*	0/16	NT
			M. rufocanus	8/37	1/8
	Solton	2007	*M. rutilus*	2/14	1/2
			M. rufocanus	1/2	0/1
			M. glareolus	0/10	NT
Altai Republic	Teletskoye Lake	2007–2009	*M. rutilus*	6/80	1/6
			M. rufocanus	1/3	1/1
			M. glareolus	1/10	0/1
Kemerovo Oblast	Balakhnino	2009	*M. rutilus*	0/5	NT
			M. rufocanus	0/6	NT
			M. glareolus	0/1	NT
	Kuzedeyevo	2007	*M. rutilus*	1/9	0/1
			M. rufocanus	0/6	NT
			M. glareolus	0/21	NT
Krasnoyarsk Krai	Parnaya	2008	*M. rutilus*	1/11	0/1
			M. glareolus	0/5	NT
	Srednyaya Shush	2008	*M. rutilus*	4/26	0/4
			M. rufocanus	9/31	0/9
Omsk Oblast	Ust-Ishim	2007	*M. rutilus*	0/11	NT
			M. glareolus	2/11	0/2
Tiumen Oblast	Kuchuk	2007	*M. rutilus*	1/29	0/1
			M. glareolus	4/42	3/4
Tomsk Oblast	Sukhorechie	2009	*M. rutilus*	1/8	0/1

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Administrative Region	Trap Locality	Trap Year	*Myodes* Species	Antibody Prevalence	Hantavirus RNA
			M. rufocanus	0/3	NT
			M. glareolus	0/3	NT

[a] Number of antibody positive *Myodes* voles/Number captured

[b] Hantavirus RNA was detected by RT-PCR, using L-segment specific primers, in lungs of anti-hantavirus antibody-positive voles

NT, samples were not analyzed

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Table 2

Oligonucleotide primers for nested PCR used in this study

Gene	Primer sequence (5′-3′)	Position[a], orientation	Reference
L	AGAGAA(G/A)T(T/C)TACTA(C/A)AAATGGAGAAATACA	20 (+)	Yashina et al., 2013
	TTTAAATT(G/A)AACAT(T/G)GC(T/C)TC(A/T)AGTGC	677 (−)	
	GA(C/T)CAGATGATAAA(G/A)CATGA(T/C)TGGTC	154 (+)	
	CTGTGTTGA(G/T)AT(A/G)TTTGA(T/G)CCATCAGT	549 (−)	
M	ATTTAA(G/A)CAATGGTG(C/T)ACTAC(T/A)AC	2644 (+)	Yashina et al., 2000
	CC(G/A)TAACACATTGC(A/G)GC	3117 (−)	
	TAGAAAGAAATGTGCATTTGC	2743 (+)	
	CCTGA(G/A)CCCCATGC(A/T/C)CCATC	3024 (−)	
S	TTCTGCAGTAGTAGTAGACTCCTTGAAAAG	1 (+ −)	
	C(A/T)GGTGCACA(G/T)GCAAANACCC	991 (−)	
	G(A/T)GG(A/C/T)CA(G/A)AC(A/T)GCAGA(C/T)TGG	380 (+)	Yashina et al., 2010 This study
	AGCTCAGGATCCATGTCATC	1271 (−)	
	CCCAGATCTATGAGTGACTTGACAGACATCCAAG	42 (+)	
	CC(A/T)GGTGT(A/G)AG(T/C)TCTTC(A/T)GC	629 (−)	

[a]The numbers correspond to genome positions of PUUV strain CG1820 (GenBank M63194, M29979, M32750).

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Table 3

Hokkaido virus (HOKV) sequences from *M. rutilus* and *M. rufocanus* and Puumala virus (PUUV) sequences from *M. glareolus* captured in Western Siberia

Trap Locality	*Myodes* species	Virus strain	GenBank accession no.			
			S segment	M segment	L segment	Host cyt *b*
Pokrovka	*M. rufocanus*	Pokrovka674/Mrf/2008	KM245964	KM245954	KM245959	KP859518
Solton	*M. rutilus*	Solton35/Mrt/2007	KM245962	KM245952	KM245957	KP859515
Teletskoye	*M. rutilus*	Teletskoye854/Mrt/2008	KM245963	KM245953	KM245958	KP859516
	M. rufocanus	Teletskoye937/Mrf/2008	KM245965	KM245955	KM245960	KP859519
Kuchuk	*M. glareolus*	Kuchuk170/Mg/2007	KP292966	KP292970	KP292967	KP859521
		Kuchuk197/Mg/2007	–	KP292972	KP292968	KP859522
		Kuchuk246/Mg/2007	–	KP292973	KP292969	KP859523

– The short 210-nucleotide sequences of the S segment (position 400–609) of PUUV strains Kuchuk197/Mg/2007 and Kuchuk246/Mg/2007 were not deposited in GenBank and were not included in the phylogenetic analysis.

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Table 4

Myodes voles with anti-hantavirus antibodies and hantavirus RNA

Trap locality	Animal no./species	Anti-hantavirus IFA titer	Hantavirus RNA[a]
Khmelevka	Khmelevka161/Mg	20	−
Kolyvan	Kolyvan721/Mrt	40	−
Pokrovka	Pokrovka613/Mrf	20	−
	Pokrovka622/Mrf	40	−
	Pokrovka630/Mrf	640	−
	Pokrovka632/Mrf	10	−
	Pokrovka670/Mrf	80	−
	Pokrovka674/Mrf	320	+
	Pokrovka678/Mrf	160	−
	Pokrovka695/Mrf	160	−
Solton	Solton35/Mrt	640	+
	Solton39/Mrt	40	−
	Solton38Mrf	40	−
Teletskoye Lake	Teletskoye338/Mrt	40	−[b]
	Teletskoye361/Mrt	40	−
	Teletskoye854/Mrt	80	+[c]
	Teletskoye860/Mrt	20	−
	Teletskoye864/Mrt	80	−
	Teletskoye2321/Mrt	80	−
	Teletskoye937/Mrf	160	+
	Teletskoye894/Mg	40	−
Kuzedeyevo	Kuzedeyevo91/Mrt	20	−
Parnaya	Parnaya1241/Mrt	80	−
Srednyaya Shush	Shush1004/Mrt	10	−
	Shush1007/Mrt	40	−
	Shush1010/Mrt	40	−
	Shush1018/Mrt	20	−
	Shush1002/Mrf	40	−
	Shush1008/Mrf	40	−
	Shush1013/Mrf	80	−
	Shush1050/Mrf	80	−
	Shush1056/Mrf	40	−
	Shush1059/Mrf	40	−
	Shush1060/Mrf	10	−
	Shush1066/Mrf	20	−
	Shush1102/Mrf	80	−
Ust-Ishim	Ust-Ishim261/Mg	20	−
	Ust-Ishim277/Mg	80	−
Kuchuk	Kuchuk170/Mg	40	+

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Trap locality	Animal no./species	Anti-hantavirus IFA titer	Hantavirus RNA[a]
	Kuchuk197/Mg	640	+
	Kuchuk207/Mg	40	−
	Kuchuk246/Mg	640	+
	Kuchuk232/Mrt	10	−
Sukhorechie	Sukhorechie1394/Mrt	10	−

[a] Hantavirus L-segment RNA was detected in rodent lungs by RT-PCR

[b] negative

[c] positive

Abbreviations: Mrt, *M. rutilus*; Mrf, *M. rufocanus*; Mg, *M. glareolus*.