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THE FAT DORMOUSE *Myoxus glis* AS A NATURAL HOST OF MEDICALLY IMPORTANT MICROORGANISMS

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The fat dormouse (*Myoxus glis*) was examined as a potential host of hantaviruses and rickettsiae. 98 animals were collected from three different regions in Slovenia (Godovič, Metlika, Snežnik). Serological methods were used for, the detection of specific viral and/or rickesttsial antibodies in animal sera samples. With the PCR method and restriction enzyme digestion, viral and rickettsial genomes were examined. *Myoxus glis* was confirmed as a host of *Hantaan* virus. The prevalence of hantaviral infection among the population of *Myoxus glis* was 13.3 %. It was established that animal age plays a role in the probability of infection with hantaviruses, but not sex. Rickettsial infection was not confirmed in *Myoxus glis* with any of the methods used.

Key words: host, *Myoxus glis*, zoonoses, natural cycle, hantaviruses, rickettsiae, Slovenia

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Istraživan je sivi puh (*Myoxus glis*) kao potencijalni domadar hantavirusa i rikecija. Sakupljeno je 98 životinja iz tri različita dijela Slovenije (Godovič, Metlika, Snežnik). Za detekciju specifičnih virusnih i/ili rikecijalnih antitijela u serumima životinja korištene su serološke metode. Pomoću PCR metode i restrikcijskih enzima istraživani su genomi virusa i rikecija. Potvrđen je *Myoxus glis* kao domadar *Hantaan* virusa. Hantaviralna infekcija postojala je kod 13.3% populacije *Myoxus glis*. Ustanovljeno je da je dob važna kod vjerojatnosti infekcije hantavirusima, a spol ne. Rikecijalna infekcija nije dokazana kod *Myoxus glis* niti jednom korištenom metodom.

Ključne riječi: domadar, *Myoxus glis*, zoonoze, prirodni ciklus, hantavirusi, rikecije, Slovenija

INTRODUCTION

Zoonoses are any infection transmitted to humans from infected animals, whether this is direct or indirect contact. The epidemiology of zoonoses depends on the frequency and nature of the contact between the vertebrate and human hosts. For better understanding of zoonoses, knowledge of their circulation in the natural environment is very important.

We have studied the fat dormouse (*Myoxus glis*) as a potential host reservoir of medically important microorganisms in Slovenia. In Slovenia, the fat dormouse is widespread and hunting dormice is an old and still living custom. People also come into contact with dormice and their excreta in houses near forests, where this animal often find shelter and some food.

We examined two zoonoses; Hemorragic Fever with Renal Syndrome (HFRS) caused by viruses in the genus *Hantavirus* of the family *Bunyaviridae* and rickettiosis of which the causative agents are bacteria in the order *Rickettsiales*.

Hantaviruses are maintained in nature primarily in rodents. They cause chronic, apparently asymptomatic infections among their reservoir hosts (LEE 1982). Infection has been found in 43 mammal species, most of them from genera *Apodemus, Rattus, Chletrionomys* and *Microtus* (TKACHENKO et al. 1983). Infection in man is a direct result of exposure to infected rodents or their infected excret (LE DUC 1987).

Rickettsia are small bacteria, obligate intracellular parasites, which are transmitted to humans by arthropods from a vertebrate reservoir (ANACKER et al. 1980).

Results of previous epidemiological and serological studies of HFRS in Slovenia indicated the simultaneous circulation of different hantaviruses among various genera of rodents (AVŠIČ-ŽUPANC 1991b). There are also data on the *Rickettsia typhi* infection of *Monopsyllus sciurorum sciurorum* fleas from the nests of the fat dormouse (TRILAR et al. 1994).

In order to determine whether *Myoxus glis* is involved in the maintenance of hantaviruses and rickettsiae in nature, their sera and organs were examined by indirect immunofluorescent assay, enzyme immunosorbent assay and polymerase chain reaction.

MATERIALS AND METHODS

Collection of samples. To get the material we were joined traditional dormouse hunters. Animals were trapped with snap traps. Fat dormice were collected during three years in different locations in Slovenia. In October 1992 in Zakrog near Godovič, in October 1994 near Metlika and in July 1995 on Mt Snežnik. In the field, laboratory samples of blood, organs (heart, lungs, liver, kidney, spleen), and biometrics data (sex, age, weight, and measures of body) of animals were taken. Organs were stored at –70 °C, sera samples were collected and stored at –20 °C.

Serological methods. For detection of specific hantaviral and rickettsial antibodies indirect immunofluorescent assay (IFA) was used. For detection of hantaviral antibodies

spot slides of Vero E6 cells infected with different hantaviruses as described previously (VAN DER GROEN et al. 1983) were used. Hantaviruses used as antigens in a study were *Hantaan* (HTN 76–118), *Puumala* (CG 18–20), and *Dobrava* (DOB 709/5). Rickettsial antibodies were detected by using commercially available reagents (Rickettsia mooseri spot and Rickettsia conorii spot, Bio Merieux). Samples were considered positive when characteristic cytoplasmic fluorescence was observed in infected cells.

An enzyme immunosorbent assay (EIA) was also used for the detection of IgG antibodies against hantaviruses as described previously (AVŠIČ-ŽUPANC 1991a).

Extraction of DNA and RNA from kidney samples. Kidneys were defrosted and 100 mg of tissue was homogenated with 1 ml TRIzol Reagent (Life Technologies Ltd, Gaithersburg, USA). Simultaneous extraction of DNA and RNA was accomplished, according to the manufacturer's procedure.

PCR assay. For detection of hantaviruses a reverse transcriptase polymerase chain reaction (RT-PCR) was used as described previously (XIAO et al. 1991). Briefly, RNA was initially reverse-transcribed to cDNA using avian myeloblastosis virus reverse transcriptase, and PCR amplification that targets a 365 bp large portion of the G2 region of the hantavirus M genome segment by using a group specific primer set (HG2F1, 5TGGGCTGCAAGTGC3 and HG2R1, 5CAACCCCAGCTAGTTTCA3') (XIAO et al. 1992).

Detection of Rickettsia-specific DNA sequences was performed in kidney samples by PCR amplification of 17 kDa antigen gene as described by WEBB et al. 1990. In brief, PCR was done by using 50 μL of the reaction mixture for 35 cycles of denaturation at 95 °C for 30 s, annealed at 57 °C for 1 min, and subjected to the sequence extension at 72 °C for 2 min (DNA Thermal Cycler, Gene Amp PCR system 9600, Perkin Elmer Cetus Corp., USA). A pair of the oligonucleotide primers (primer 1, 5'GCTCTTGCAACTTCTATGTT3'; primer 2, 5'CATTGTTCGTCAGGTTGGCG3') was synthesised on the basis of the DNA sequence for the 17 kDa protein antigen from *R.rickettsii* (ANDERSON et al. 1987, ANDERSON & TZIANABOS 1989). Each of the 20-base oligomer primers is complementary to a region of DNA in *R.rickettsii, R.conorii, R.prowazekii, R.typhi* and the ELB agent where the nucleotide sequences are very similar (ANDERSON et al. 1987, ANDERSON & TZIANABOS 1989, ADAMS et al. 1990). The length of the rickettsial genome target for amplification was predicted to be 434 bp (WEBB et al. 1990). Precautions to avoid PCR product carryover and sample-to-sample contamination were rigorously taken (KWOK & HIGUCHI 1989). The different steps of the PCR procedure were performed in separate rooms with different positive-displacement pipette sets. Amplified DNA was visualised by electrophoresis on 3% agarose gels containing 0.5 μL/mL ethidium bromide.

Restriction analysis of PCR products. The identity of PCR products obtained by using HG2F1 and HG2R1 primers was determined by restriction endonuclease digestion: 10 1 of each PCR product was mixed with 5 to 10 units of each enzyme (Rsa I, Hinf I, and Alu I) in a final volume of 20 μL and incubated at 37 °C for 1 h. Fragments were size-fractionated by electrophoresis on 3% agarose gels contain-

Table 1. Standard restriction pattern of the digested 365 bp large fragment of hantavirus genome

	Hantaviruses		
Restriction enzymes	HTN	PUU	DOB
Rsa I	288 77	365	170 123 72
Hinf I	365	204 161	195 170
Alu I	263 93	224 122	304 61

Abbreviations: HTN – Virus *Hantaan*, PUU – Virus *Puumala*, DOB – Virus *Dobrava*.

ing 0.5 μL/mL ethidium bromide. The restriction pattern was analysed by comparison with the pattern of representative strains of each hantavirus as previously shown by XIAO et al. 1992 (Table 1).

RESULTS

Sera and kidney tissues were obtained from 98 fat dormice (46 female and 52 male) trapped at three study sites during 1992 to 1995 (Fig. 1). All sera were exam-

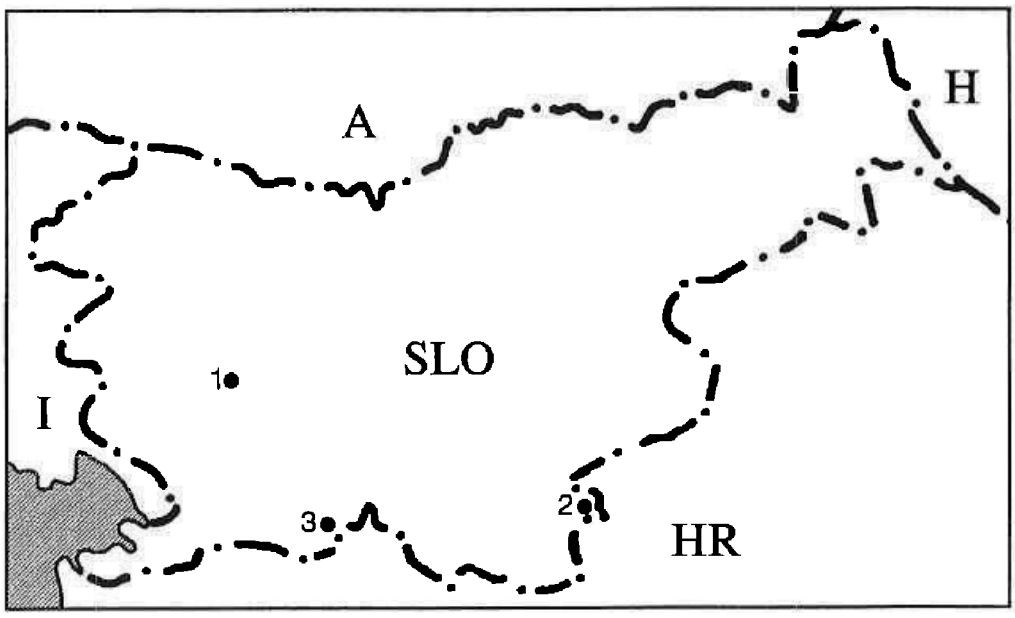

Fig. 1. Map of collecting sites: 1 – SLO: Godovič, Zakrog, 2 – SLO: Metlika, Drašiči, Mačkov vrh, 3 – SLO: Snežnik, Sviščaki

ined with IFA for the presence of hantaviral and rickettsial IgG antibodies and there were no positive results. However, when the EIA method was used, hantaviral antibodies were found in 5 (5.1%) sera samples. The hantavirus-positive fat dormice were from Godovič (3 of 36; 8.3%), and Snežnik (2 of 21; 9.5%). All 5 sera showed reactivity with *Hantaan* antigen (HTN 76–118) and not with *Puumala* (CG 18–20) or *Dobrava* (DOB 907/5) antigens (Table 2).

RNAs extracted from kidney tissues were amplified with hantavirus specific primers HG2F1 and HG2R1. Out of 98 RNA samples the predicted 365 bp PCR product was detected in 10 (10.2%). Hantavirus-positive fat dormice were from Godovič (4 of 36; 11.1%), and Metlika (6 of 41; 14.6%) (Table 2). Since primers HG2F1 and HG2R1 are hantavirus genus specific, PCR products were digested to determine virus type. The digestion pattern of PCR positive samples were homologous to those seen with *Hantaan* virus (Fig. 2).

Table 2. Prevalence of hantaviruses infection in dormice by using serological and molecular methods

			EIA			Molecular methods	
Location	No. of sample	Sex	HTN	PUU	DOB	PCR for genus *Hantavirus*	RE
Godovič N = 36	93/92		M	+	–	–	–
	98/92		F	+	–	–	+
	106/92	M	–	–	–	+	HTN
	107/92	F	–	–	–	+	HTN
	116/92	M	+	–	–	+	HTN
Total			3	0	0	4	4
Metlika N = 41	20/94	M	–	–	–	+	HTN
	33/94	M	–	–	–	+	–
	40/94	M	–	–	–	+	–
	41/94	F	–	–	–	+	HTN
	45/94	M	–	–	–	+	–
	52/94	F	–	–	–	+	–
Total			0	0	0	6	2
Snežnik N = 21	167/95	F	+	–	–	–	–
	178/95	F	+	–	–	–	–
Total			2	0	0	0	0
Total N = 98			0	0	0	10	6

Abbreviations: M – Male, F – Female, EIA – Enzyme Immuno Assay, PCR – Polymerase Chain Reaction, HTN – Virus *Hantaan*, PUU – Virus *Puumala*, DOB – Virus *Dobrava*, RE – Restriction Enzyme Digestion

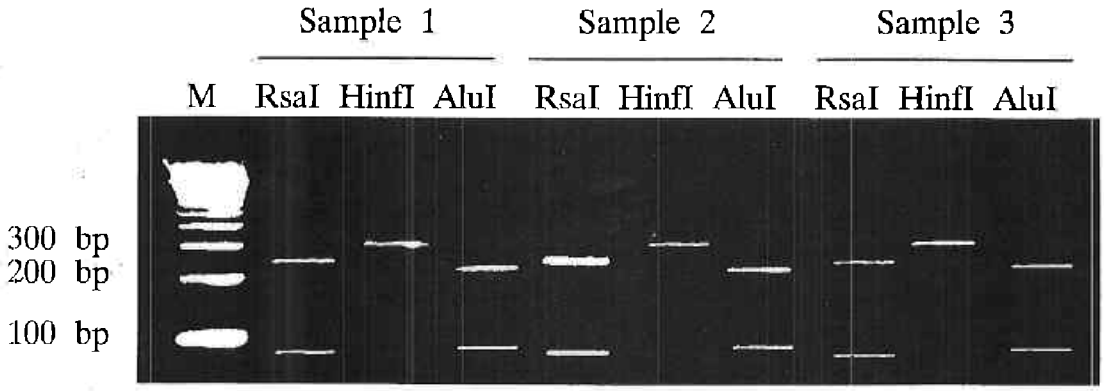

Fig. 2. Restriction pattern of a 365 bp large fragment of *Hantaan* virus with Rsa I, Hinf I and Alu I restriction enzymes

Therefore, the overall infectivity rate of hantaviral infection among fat dormice in Slovenia is 13.3%. The distribution of infected animals varies from 19.4% in Godovič, 14.6% in Metlika, and 9.5% in Snežnik. Sex distribution in the captured population was 46 female (46.9%) and 52 male (53.1%). Sex distribution of infected animals was 6 female (46.1%) and 7 male (53.9%) (Table 2).

The DNA extracted from dormouse kidney samples was amplified with primers 1 and 2 (17-kDa gene) which are *Rickettsia* genus specific and produce a 434 bp product. In none of the DNA samples the predicted 434 bp product was detected. Both serological and molecular methods fail to prove the occurrence of rickettsiae in fat dormice in Slovenia.

DISCUSSION

Hemorragic Fever with Renal Syndrome (HFRS) caused by hantaviruses is a zoonotic infection that is endemic in Slovenia. During the last ten years broad clinical, virological and epidemiological studies have been conducted in the country. Results of previous epidemiological studies of hantavirus natural focies in Slovenia indicated the simultaneously circulation of different hantaviruses among various genera of rodents (AVŠIČ-ŽUPANC 1991b). In order to determine whether weather *Myoxus glis* is involved in the maintenance of hantaviruses in nature, their sera samples and kidney tissues were examined for the presence of specific antibodies and viral genome, respectively. Hantaviruses infection of natural reservoirs is commonly studied by using serological methods (GROEN et al. 1995, MA et al. 1995). When IFA and EIA methods were used in our study specific hantaviral antibodies were found in 5 (5.1%) animals. Results of our previous seroepidemiological studies that involved mainly field and wood mice and bank voles represent a seroprevalence of from 14.7% to 29.4%, depending on the study site (AVŠIČ-ŽUPANC et al. 1993). When a molecular method, RT-PCR, was used for direct detection of a portion of hanta-

virus genome in animal tissues a two times higher (10.2%) infectivity rate was found. This phenomenon was already seen in one of our initial epidemiological studies where the prevalence of virus antigen was demonstrated by less sensitive assays, like IFA and EIA (AVŠIČ-ŽUPANC et al. 1991b). Similar observations were reported by other groups (GROEN et al. 1983, LE DUC et al. 1987). From our results we could notice that in the same individuals there were no specific antibodies, whereas the viral genome was detected (Table 2). One explanation is low the sensitivity of our serological tests because secondary antibodies used in both IFA and EIA methods were not species-specific (e.g. anti-myoxus). We have to assume the possibility that animals could be captured before or in the beginning of seroconversion. In the other hand, there were cases where we could not detect the viral genome but there were specific antibodies present. We predicted that those animals were infected some time ago; altough the virus was already cleared out they may remain seropositive throughout their lives. It should be considered that knowledge of hantavirus infection in small mammals in their natural environment is not complete.

With both serological and molecular methods there was 13.3% prevalence of hantaviruses infection among *Myoxus glis* in Slovenia and this is compatible with the results from our previous studies where the overall infectivity was 12% (AVŠIČ-ŽUPANC et al. 1993). The sexual structure of captured animals was 46.9% female and 53.1 % male. Among infected animals the sexual structure was 46.1% female and 53.9 % male. These results provide evidence that the sex of animal does not influence the probability of hantaviral infection. But we assume that there is a connection between the age of the animal and the probability of infection. Namely, the lowest infectivity rate (9.5%) was found in animals from Snežnik (results from Godovič and Metlika were 19.4% and 14.6%) where all dormice were in the juvenile stage, from the present spring. Those animals had been exposed to the environment for a shorter time and were not yet mating. Mating includes behaving patterns (territory marking, more mobility, fights), thus suggesting a greater probability for hantaviral infection.

Our work confirms previous observations (AVŠIČ-ŽUPANC et al. 1993) that *Myoxus glis* is a potential natural reservoir of hantaviruses. The results of the present study are certified not only with serological but also with more reliable methods of molecular virology (RT-PCR). Furthermore, restriction enzyme digestion of PCR products revealed that the hantavirus present in dormice in Slovenia is *Hantaan*. Since the oligonucleotide primers used in RT-PCR reaction could amplify any of the virus types in the genus *Hantavirus* it was necessary to select a typization strategy afterwards. Poljak has shown in his study of hantavirus typization after the PCR method (POLJAK 1993) that certain restriction enzymes are sufficient to differentiate all hantaviruses circulating in Slovenia. Among human cases of HMRS in Slovenia there was also a traditional dormouse hunter. Although among them severe forms of HMRS are rare, testing their sera for hantaviral antibodies could be interesting. It would be interesting to know if infections are really rare or whether there are more subclinical cases of disease that hide the frequency of infection.

By using serological and molecular methods we were unable to prove rickettsial infection in dormice. Also the enzootic cycle involving *Myoxus glis* and *Monopsyllus sciurorum sciurorum* was not demonstrated. To provide evidence that dormice are possible reservoirs for rickettsia we suggest a study of experimental infection.

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SUMMARY

Fat dormouse *Myoxus glis* as a natural host of medically important microorganisms

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Zoonoses are any infection transmitted to humans from infected animals, whether this is direct or indirect contact. The epidemiology of zoonoses depends on the frequency and the nature of the contact between the vertebrate and the human hosts. For a better understanding of zoonoses, knowledge of their circulation in the natural environment is very important. Therefore, our study included the fat dormouse (*Myoxus glis*) as a potential host reservoir of medically important micro-organisms in Slovenia. Two zoonoses were examined; Hemorrhagic Fever with Renal Syndrome (HFRS) caused by viruses in the genus *Hantavirus* of the family *Bunyaviridae* and rickettsiosis of which the causative agents are bacteria in the order Rickettsiales. Hantaviruses are maintained in nature primarily in rodents and insectivores. They cause chronic, apparently asymptomatic infections among their reservoir hosts. Infection in man is a direct result of exposure to infected rodents or insectivores or their infected excreta. *Rickettsia* are small bacteria, obligate intracellular parasites, which are transmitted to humans by arthropods from a vertebrate reservoir. Results of previous epidemiological and serological studies of HFRS in Slovenia indicated the simultaneous circulation of different hantaviruses among various genera of rodents. Our former results also revealed a natural cycle involving *Rickettsia typhi* infection of *Monopsyllus sciurorum sciurorum* fleas from the nests of the fat dormouse. To elucidate the variability of hantaviruses spreading by their natural hosts and to prove the new natural cycle of *R. typhi*, fat dormice were collected during three years in different locations in Slovenia (Godovič, Metlika, Snežnik). Indirect immunofluorescence assay (IFA) and enzyme immunoassay (EIA) were used as serological methods for the detection of specific hantaviral and rickettsial antibodies in dormice sera. Recent advances in the polymerase chain reaction (PCR) technology that allow the detection of viral RNA or bacterial DNA in tissue samples were applied to the study of naturally infected animals. For hantavirus typing strategy restriction endonucleas digestion was used. With thouse of serological and molecular methods, the overall prevalence of hantavirus infection among *Myoxus glis* in Slovenia was 13,3%. Out of 98 examined samples, five had specific hantaviral antibodies and viral RNA was detected in ten animal kidney tissues. When PCR products of hantaviral positive animals were digested with 5 restriction enzymes the cleavage patterns revealed that the fat dormouse in Slovenia is a reservoir of *Hantaan* virus. Neither the serological or the molecular methods used in the study to detect specific antibodies to *R. typhi* and/or to demonstrate rickettsial DNA in animal tissue samples to proved that *Myoxus glis* serves as a reservoir in the natural cycle of *R. typhi*. Therefore we conclude that there is considerable evidence that the fat dormouse *Myoxus glis* is a reservoir of hantavirus *Hantaan* in Slovenia. Since *Hantaan* virus is a causative agent of a very severe form of HFRS, with 5–10% fatality, it is important to be aware of this infection while hunting dormice.

SAŽETAK

Sivi puh *Myoxus glis* kao domadar medicinski važnih mikroorganizama

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Zoonoze su sve infekcije koje ljudima prenose zaražene životinje, bilo direktnim ili indirektnim kontaktom.

Epidemiologija zoonoza ovisi o učestalosti i prirodi kontakta kralježnjaka i ljudskog domadara. Za bolje razumijevanje zoonoza važno je znati kako one kruže u prirodnom okolišu. Zato smo u naše istraživanje uključili sivog puha (*Myoxus glis*) kao potencijalni rezervoar medicinski važnih mikroorganizama u Sloveniji. Ispitivane su dvije zoonoze; hemoragična groznica s bubrežnim sindromom (HFRS) koju uzrokuju virusi iz roda *Hantavirus* iz porodice *Bunyaviridae* i rikecioze, čiji su uzročnici bakterije iz reda Rickettsiales. Hantavirusi u prirodi uglavnom žive u glodavcima i kukcojedima. Uzrokuju kronične, očito asimptomatičke infekcije u svojim domadarima koji im služe kao rezervoari. Infekcija čovjeka je direktna posljedica kontakta sa zaraženim glodavcima ili kukcojedima ili njihovim zaraženim ekskretima. *Rickettsia* su male bakterije, obligatorni intracelularni paraziti, koji se na ljude prenose putem člankonožaca i to od kralježnjaka koji služe kao rezervoar. Rezultati prijašnjih epidemioloških i serološkiх studija HFRS u Sloveniji ukazivali su na simultano kruženje različitih hantavirusa među različitim rodovima glodavaca. Naši prijašnji rezultati otkrili su također i prirodni ciklus u kojem se radilo o *Rickettsia typhi* infekciji kod buhe *Monopsyllus sciurorum sciurorum* iz gnijezda sivog puha. Da bi se razjasnila varijabilnost hantavirusa koji se šire putem svojih prirodnih domadara i da bi se dokazao novi prirodni ciklus *Rickettsia typhi*, tijekom tri godine prikupljeni su sivi puhovi s različitih mjesta u Sloveniji (Godovič, Metlika, Snežnik). Kao serološke metode za detekciju specifičnih hantavirusnih i rikecijalnih antitijela u serumu puhova korištene su metoda indirektne imunofluorescencije (IFA) i imunoenzimska metoda (EIA). Za proučavanje prirodno zaraženih životinja korištena je uznapredovala lančana reakcija polimeraze (PCR) koja omogućava detekciju viralne RNA i bakterijske DNA u uzorcima tkiva. Za tipiziranje hantavirusa korištena je digestija restrikcijskim endonukleazama. Serološkim i molekularnim metodama dokazana je prisutnost infekcije hantavirusima kod *Myoxus glis* u Sloveniji od 13.3%. Od 98 pregledanih uzoraka, kod pet su utvrđena specifična antitijela na hantavirus, a viralna RNA je dokazana u deset uzoraka tkiva iz bubrega životinja. PCR produkti životinja pozitivnih na hantavirus bili su zatim obrađeni s 5 restrikcijskih enzima i mjesta loma otkrila su da je sivi puh u Sloveniji rezervoar *Hantaan* virusa. I serološke i molekularne metode korištene u ovom istraživanju za dokazivanje specifičnih antitijela na *R. typhi* i/ili prisutnost rikecijalne DNA u uzorcima životinjskog tkiva, nisu uspjele dokazati da *Myoxus glis* služi kao rezervoar u prirodnom ciklusu *R. typhi*. Zato zaključujemo da postoje značajni dokazi o sivom puhu *Myoxus glis* kao rezervoaru hantavirusa *Hantaan* u Sloveniji. Budući da je *Hantaan* virus uzročnik vrlo opasnih oblika HFRS, sa smrtnošću 5–10%, važno je prilikom lova na puhove čuvati se te infekcije.