

SHORT REPORT: DUAL INFECTIONS WITH *PUUMALA* VIRUS AND *LEPTOSPIRA INTERROGANS* SEROVAR LORA IN A BANK VOLE (*CLETHRIONOMYS GLAREOLUS*)

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Abstract. Leptospirosis and hemorrhagic fever with renal syndrome are public health problems in Croatia. Diagnosis and epidemiology of these diseases are complicated because these two diseases are sympatric in certain areas. We describe a natural dual infection of Puumala virus and a leptospire in a bank vole (*Clethrionomys glareolus*).

Hantaviruses, which are members of a genus within the family Bunyaviridae, can cause either of two human zoonoses: hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS).¹ Leptospirosis, also a zoonotic infection, is caused by pathogenic members of the bacterial genus *Leptospira*.² Both etiologic agents are rodent-borne and are maintained in persistently infected small rodents. Transmission from reservoir hosts to humans is possible through inhalation of virus-contaminated aerosol of rodent excreta (hantaviruses)¹ or direct or indirect contact with contaminated environment (leptospira).³ Farmers, soldiers, hunters, campers, hikers, veterinarians, and laboratory workers have the highest risk of infection. The widespread geographic distributions of rodents harboring these pathogens indicate considerable disease-causing potential essentially worldwide. Dual infection of these two agents in humans has been described previously.^{4,5} We have reported dual infection with Dobrava virus (DOBV) and *Leptospira interrogans* serovar hardjo in one patient.⁵

During September 2002, we trapped rodents in northeastern Croatia at two sites: Okučani (45°22'28"N, 17°17'05"E) and Nova Gradiška (45°18'30"N, 17°17'10"E) (approximately 20 km apart) (Figure 1), which were recently shown to be natural foci of HFRS.⁶ The same region in northeastern Croatia is also known as an area where human and animal leptospirosis are common.⁷ Sampling of small rodents was done at a hillside on Psunj mountain, ≤ 400 meters above sea level, in forests where common beech (*Fagus sylvatica* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) predominate. Rodents were captured using Sherman live traps (H.B. Sherman Traps, Tallahassee, FL) and snap traps. We followed animal experimentation guidelines approved by the American Society of Mammalogists.⁸ Rodents were identified to species, humanely killed, and dissected aseptically, with kidney and lung tissues collected for further studies. Small 50-mg pieces of frozen lung tissues were used for immunoblotting and kidney tissue was extracted for detection of hantaviral RNA. A Western blot was used to test for the presence of hantaviral nucleocapsid antigen. Homogenized specimens were sepa-

rated by electrophoresis and immunoblotted with rabbit polyclonal antibodies against Puumala virus (PUUV) and DOBV recombinant antigen. A swine anti-rabbit antibody conjugated to horseradish peroxidase was used as the secondary antibody and bands were stained with diaminobenzidine. Total RNA was extracted from kidney tissue of antigen-positive rodent(s) with TRIzol reagent (GIBCO-BRL, Gaithersburg, MD) following the manufacturer's instructions. Hantaviral RNA in antigen-positive rodents was detected in their tissues by reverse transcription–polymerase chain reaction (PCR) using the cross-reactive outer primers MOF103 and MOR204, which amplify a 490-basepair region from the M segment (encoding G1) of a number of different hantaviruses, as previously described.⁹ A second-round PCR (for nucleotides 1296–1620) was done using PUUV-specific primers G1F (5'-GTGTCCAGAGATTCCGTGGT-3') and G1R (5'-GAAC-ATAAGTATGCGAATGCGAATGCAA-3') previously described,¹⁰ which reside within the amplified region of the RT-PCR primers in a nested fashion. All PCR products were sequenced automatically using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, CA).

Kidney tissue also was used for culture and recovering the isolates of leptospiral strains. After a first typing to determine serogroup characteristics by microscopic agglutination using rabbit antisera, we differentiated serovars. The intact genomic DNA was extracted from leptospiral isolates, digested with *Sgr* AI, and subjected to pulsed-field gel electrophoresis. DNA fragments were separated according to their sizes. Leptospire with identical restriction fragment patterns were considered the same serovar.

Rodents captured included bank voles (*Clethrionomys glareolus*, n = 57), yellow-necked field mice (*Apodemus flavicollis*, n = 35), and long-tailed field mice (*Apodemus sylvaticus*, n = 13). PUUV antigen was detected by Western blot in one female bank vole of a total 33 bank voles captured at Nova Gradiška (Figure 1). Hantaviral RNA was extracted from kidney tissue of the antigen positive rodent. The PCR product authenticity was confirmed by sequence analysis.

Leptospiral DNA also was obtained from the same vole. Serotyping by microscopic agglutination using rabbit antisera and genetic characterization by digestion with a restriction enzyme, followed by pulsed-field gel electrophoresis, showed

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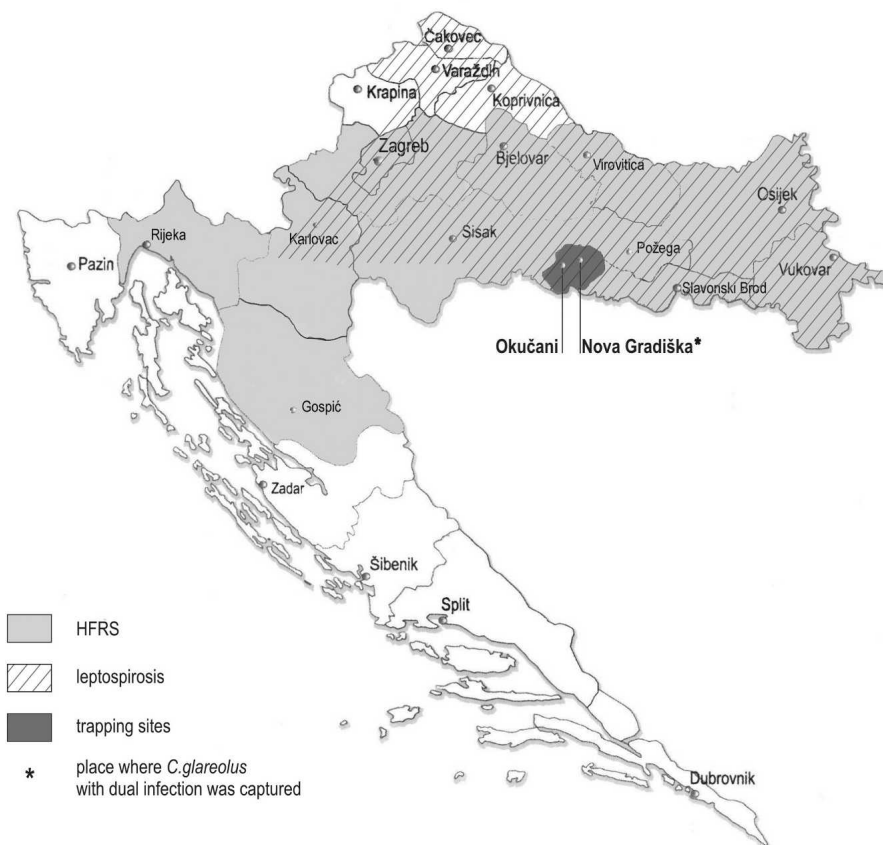


FIGURE 1. Geographic distribution of hantaviral infections and leptospirosis in Croatia. *Clethrionomys glareolus* with dual infection was captured at trapping site Nova Gradiška (45°18'30"N, 17°17'10"E). HFERS = hemorrhagic fever with renal syndrome.

that this leptospire was most closely related to the genotype *L. interrogans sensu stricto* serogroup *Australis* serovar lora.

Except for the Adriatic coastal and islands, all of Croatia has been found to be endemic for HFERS.¹¹ During HFERS outbreaks in Croatia, small, focal, disease-endemic areas with a high prevalence of antibody to hantaviral antigens were reported at Mala Kapela mountain,¹² Novska,¹³ and Dinara mountain.^{11,14} In the last 2002 HFERS outbreak, a newly recognized disease-endemic area was confirmed near Nova Gradiška (in northeast Croatia).⁶ In addition, a small survey of rodents showed that the entire area of Posavina in northeastern Croatia was a wide natural focus of leptospires (Figure 1).^{7,15}

The bank vole is considered to be the principal reservoir of PUUV in Croatia, with yellow-necked field mice, long-tailed field mice, and striped field mice (*Apodemus agrarius*) playing minor roles in maintenance and transmission of hantaviruses.^{11,12} Coincidentally, rodents most commonly found with leptospires are these same four species.¹⁵ Serovar lora has been isolated from yellow-necked field mice and genetically characterized in Croatia.⁷

We found one bank vole captured near Nova Gradiška with a dual infection of PUUV and *L. interrogans* serovar lora (Figure 1). Since these pathogens share the same reservoir host, this suggests that their geographic distribution provides opportunity for individual bank voles to be infected with both pathogens.

Hantaviruses are principally associated with single rodent

hosts.¹⁶ There are no data on the prevalence hantavirus/leptospirosis dual infections among rodents. It is also not known what would be the risk for humans to encounter both infections at the same time. To the best of our knowledge, our study is the first to demonstrate co-infection of a hantavirus and a leptospira in a rodent reservoir host. From previous studies it is known that northeastern Croatia has a status of a combined focus of leptospirosis and hantavirus infection (Figure 1).^{6,7,15} We showed that dual infection with these two pathogens may occur in bank voles under natural conditions. Therefore, determining the presence and spread of these etiologic agents of human illnesses by testing local rodent populations might predict the potential for disease emergence. We suggest that tests for both pathogens be used for diagnosis of illnesses in patients suspected to have HFERS or leptospirosis in Croatia and elsewhere in the region.

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