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Detection of Puumala virus in the tissue of infected naturally rodent hosts in the area of central Dinarides

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A B S T R A C T

Hantaviruses are the causative agents of haemorrhagic fever with renal syndrome (HFRS) in Euroasia and of hantavirus cardiopulmonary syndrome (HCPS) in the North, Central and South America. HFRS is endemic in the Balkan Peninsula, where sporadic cases or outbreaks have been reported. Puumala virus (PUUV) is the causative agent of nephropathia epidemica (NE), a mild form of HFRS. PUUV is carried by the bank voles (*Myodes glareolus*). In this study, we investigated viral RNA from 76 tissues samples (lung $n = 30$, heart $n = 6$, liver $n = 18$ and kidney $n = 22$) of infected naturall rodent hosts in the area of Central Dinarides caught in live traps. Puumala virus was extracted from 34,7% (16/46) rodents by nested reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR. Overall, 18 (21,4%) specimens of internal organs (kidney $n = 8$, liver $n = 6$, heart $n = 2$ and lung $n = 2$) were positive for PUUV. It was shown a high rodent infestation rate in a relatively low number of rodent and their organs, although mice were not caught during the time of high density population of host rodents.

1. Introduction

PUUV belongs to the genus *Hantavirus*, family *Bunyaviridae* that is comprised of five genera: *Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus* and *Tospovirus* (Nichol et al., 2005). All viruses in these genera have a similar virion structure, genome organization and replication strategy. Their genome has 3 molecules of negative or ambisense single strand RNA and it consists of three segments: the L (large) encoding the viral polymerase protein; the M (middle) encoding a glycoprotein precursor; and the S (small) encoding the nucleocapsid protein (N) (Schmaljohn et al., 1996). All hantavirus- related diseases are caused by Hantaan (HTN), Dobrava/Belgrade (DOB/BEL), Seoul (SEO), and Puumala (PUU) virus, showing different degrees of fever and renal involvement, with or without hemorrhagic manifestations, collectively referrred to as HFRS (Lee et al., 1989; Papadimitriou, 1995).

The natural hosts of Hantaviruses are small mammals. Hantaviruses are host-specific and are primarily associated with unique rodent hosts. Therefore, the distribution of a specific virus closely correlates with the geographical distribution of its natural host species (Plusnin et al., 1996). In nature, hantaviruses are maintained persistently infected in rodents or insectivores. The mechanism of persistent hantavirus infection in natural hosts is poorly understood.

The natural host of PUUV, bank vole *Myodes glareolus,* is widely distributed throughout Bosnia and Herzegovina (B&H). Specimens from three genuses were found in B&H; Apodemus, Microtus and Myodes, where is dominanting species *M. glareolus* (Schreber, 1780) (Muzaferović et al., 2009.). According the revised list of mammals (Mammalia) in B&H, species is classified into Classis: Mammalia, Order: Rodentia (Bowdich, 1821), Familia: Cricetidae (Fischer, 1817), Genus: Myodes (Pallas, 1811). This genus is known as Clethrionomys (Tilesius, 1850) (Wilson and Reeder, 2005). *M. glareolus* on the territory of B&H is described as subspecies of *Clethrionomys glareolus bosniensis* (Martino, 1945) (type locality: Lah near Sarajevo).

M. glareolus which inhabits not only wet coniferous and mixed forests, but also leafed forest, river banks and marshy areas, is widely spread throughout B&H. Since 1952, B&H has been recognized as a highly endemic region for hantavirus infections (Hukić and Šibalić, 2003).

In contrast to pathogenic human infections, hantaviruses are generally believed to infect natural hosts in a persistent fashion and are nonpathogenic to them as their reservoir hosts (Gavrilovskaya et al., 1990., Yanagihara et al., 1985a,b). Although an immune

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response to the virus is induced, the natural hosts harbor viral RNA and antigens, especially in the lungs, and maintain the virus for more than one year after infection (Lee et al., 1981). The virus is shed in rodent urine, feces and saliva, which are believed to represent the major source of hantavirus infections in humans through inhalation (Lee et al., 1981; Vapalahti et al., 2003; Yanagihara et al., 1985a,b).

The mechanisms for persistent infection of hantaviruses in their natural hosts remains unclear. One factor that impedes the clarification of these mechanisms is a lack of animal models.

Similar studies have been done in the neighboring countries. Tadin A. et al., based their stady on nested RT-PCR, by which Puumala virus (PUUV) RNA was detected in 41/53 (77,4%) bank voles *(M. glareolus)*(Tadin et al., 2014.). A very large bank-vole population combined with an extremely high infection rate of PUUV was responsible for this unusual winter outbreak of HFRS in Croatia.

The aim of the study was to detect Puumala virus in the tissue of infected naturally rodent hosts in the area of Central Dinarides during the 2009 by nested reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR.

2. Materials and methods

The research protocol was approved by Scientific Council of the Faculty of Sciences in Sarajevo.

Bank voles were trapped during the 2009 with live-traps at three localities around Central Dinarides: Igman, Zvijezda and Bitovnja mountains.

Morphometric parameters including body weight, length, tail length, foot and ear shells were used for identification of the small rodent species *M. glareolus* In total, 76 internal organs (hearts, lungs, livers and kidneys) were collected from 46 *M. glareolus* rodents and tested or stored at −70 °C until analysis. To identify species *M. glareolus* captured small rodents were analysed with morphometric parameters including, body weight, length, tail length, foot and ear shells.

Their internal organs (heart, lung, liver and kidney) were collected and stored at −70 °C until analysis.

The internal organs were treated for RNA preparation at the Institute of Clinical Microbiology, Department of virology, University Clinical Center Sarajevo including laboratory work which requires a biosafety level 3. With work in this closed system, generation of contaminating aerosols was avoided.

Quantity of the 10–50 mg of the tissue of an each organ was homogenized (Kontes PELLET PESTLE® Micro Grinders) to perform total RNA extraction using the QIAamp Viral Mini Kit (Qiagen, Hilden, Germany) or Trizol LS Reagent procedure (Invitrogen Life Technologies,Life Technologies TM125, Carlsbad, NM, USA) according to the manufacturer's instructions. For the real time RT-PCR assay, the AMBION AgPath-ID One-Step RT PCR Kit (Applied Biosystems, Rochev, Branchburg, New Yersey, USA) was used with the ABI Prism 7700 (Applied Biosystems) instrument.

The primers and probe were designed based on the aligment of the S segment sequences (Avšič-Županc et al., 2007). Total volume of the reaction mixture of 25 μl contained: 5 μl of extracted RNA, 12,5 μl of One Step RT PCR Master Mix, 0,3 μl (50 μM) of the forward primer PUU D (5′- ACATCATTTGAGGACAT-3′), 0,3 μl (50 μM) of the reverse primer PUU L (5′GGAGTAAGCTCTTCTGC-3′), 0,25 μl (200 nM) wild type (wt) PUUS probe (5′(TET)TCCATGCCAACAGCCCAGTCAAC(DABCYL)-3′) and 0,5 μl (20 μM) of internal control (IC) (5′(FAM)ACTGTTCGTTGAGCGA(DABCYL)-3). Other reagents in the reaction were added as instructed by manufacturer.

Table 1
Summary of the rodent specimens findings to the presence of the PUUV.

	Real time RT PCR				Nested RT PCR			
Specimen	Kidney	Lung	Liver	Heart	Kidney	Lung	Liver	Heart
PUUV positive	8	2	4	2	0	0	2	0
PUUV negative	8	26	10	2	6	2	2	2
Total	16	28	14	4	6	2	4	2
%PUUV positive	50	7,14	28,5	50	0	0	50	0

Reverse transcription was initiated at 45 °C for 10 min, followed by PCR activation at 95 °C for 10 min and 45 cycles of a two-step incubation at 95 °C for 10 s and 53 °C for 45 s.

As a positive control for real-time RT-PCR, RNA extraction the culture of Vero E6 cells infected with Puumala virus type CG 18–20 (Russia) was performed. Puumala type CG 18–20 (Russia) was isolated from a *M. glareolus* trapped in the Bashkiria region in Russia. RNAse free water was used as a negative control.

The reverse transcription (RT) was performed according to the manufacturer's instructions (Promega, Access RT-PCR System, Promega Corporation, Madison, USA) to obtain cDNA. The primary PCR using the outer primers to the viral genome segment PPT334C (5′-TATGGIAATGTCCTTGATGT-3′) and PPT 986R (5′-GCACAIGCAAAIACCCA-3′) (Bowen et al., 1997; Nichol et al., 1993) generated 653 bp amplicons.

The thermal cycling conditions were as follows: inactivation of reverse transcriptase by Tfl polymerase activation at 94 °C for 2 min, and proceeded with the 40 cycles of the primary PCR: 30 s at 94 °C, 1 minute at 45 °C, 2 min at 68 °C, one stage at 68 °C for 7 minutes and hold at 4 °C. Total volume of each reaction was 50 μl consisting of 10 mM dNTPs, 25 mM $MgSO_4$, 5 U/μl of TFl polymerase (Jena Bioscience, GmbH, Germany), 50 μM of oligonucleotide primers, AMV/Tfl 5X Reaction Buffer and 5 μl RNA.

The quality of the RT-PCR products of the target length was checked by 2% agarose gel electrophoresis in 2 ul Loading Dye Solution buffer (LB buffer) for 45 min at 140 V, and then visualized in UV transilluminator. Nested (secondary) PCR was done using the inner primer set PPT376C S segment (5'-CCI AGT GGI CAI ACA GC-3′) and PPT716R (5'-AAI CCI ATI ACI CCC AT-3') generating amplicons of 341 bp in size (Nichol, 1993., Bowen, 1997). Thermal cycling conditions were as follows: 94 °C for 2 min, 36 cycles of steps 94 °C for 30 s, 45 °C for 1 min, and 68 °C for 2 min, ending with 7 minutes incubation at 68 °C. PCR took place in 50 μl volume containing 10 mM dNTPs, 25 mM $MgCl_2$, 5 U/μl of TFl polymerase (Jena Bioscience, GmbH, Germany), 50 μM of oligonucleotide primers, 10X Reaction Buffer (Jena Bioscience, GmbH, Germany) and 1 μl of the primary PCR product. The 10 μl of the final (secondary) PCR products were analyzed by 2% agarose gel electrophoresis as it is described above. As a positive control for nested RT-PCR, the RNA PUUV mark R3487 was used.

3. Results

In total, 76 mice specimens were tested by nested RT PCR and real time RT-PCR assay. The results are summarized in Table 1.

Puumala virus was identified in 34,7% (16/46) rodents and in 23,1% (18/76) organs by real time RT-PCR. The nested RT-PCR was performed for 14 samples (lung $n = 2$, kidney $n = 6$, liver $n = 4$ and heart $n = 2$) and in 14,3% (2/14) fragment of expected size (341 bp) was observed (Fig. 1).

The presence of PUUV RNA was also examined in 30 lung, 6 heart, 18 liver and 22 kidney samples. Viral RNA was successfully detected in 23,1% (18/76) organs (2 lungs, 6 livers, 2 hearts and 8 kidneys) (Table 1).