

Failure to detect viral RNA in bat samples collected in the Balkan region

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Summary. Bats represent a known reservoir of emerging viruses, yet no molecular data are found about the occurrence of zoonotic viruses in bats in the Balkans. The aim of this study was to determine the presence of paramyxo- and hanta-viruses in bats, examined by PCR in 95 deceased bats, that were collected in Serbia and Montenegro, during the period 2002 to 2009. All samples tested positive for beta-actin mRNA, confirming successful RNA isolation and amplification. However, no sample tested positive for virus specific RNA. Our findings might reflect tissue degradation in carcass samples and do not exclude bats as potential viral reservoir in the surveyed geographic area.

INTRODUCTION

Bats can be host to a range of zoonotic and potentially zoonotic pathogens (Kohl & Kurth, 2014). In addition to rabies (RABV) and other lyssaviruses, bats have been identified as the likely reservoir for coronaviruses and filoviruses. Recently, a new distinct lineage of influenza A virus has been discovered in little yellow-shouldered bats (*Sturnira lilium*, family Phyllostomidae) in the Americas (Tong *et al.*, 2012). Close relatives of human hepatitis viruses B and E have been described in bats over the last years, whereas a range of bats associated viruses also include adenoviruses, astroviruses, bornaviruses, herpesviruses, papillomaviruses, reoviruses, retroviruses and in particular hantaviruses and paramyxoviruses (Hayman *et al.*, 2013; Drexler *et al.*, 2012a; Drexler *et al.*, 2013; Hun Gu *et al.*, 2014; Drexler *et al.*, 2014).

Serbia is a southern-European country situated on the Balkan peninsula, known as a gateway between Africa, Middle-East and Europe (Figure 1). Hantaviruses are known

to be endemically present in the Balkan region, including Serbia, both in humans and in animal reservoirs comprising several rodent species and causing hemorrhagic fever with renal syndrome (Gligic *et al.*, 1992a). On the other hand, paramyxoviruses are widely distributed worldwide and known to be associated with a range of human diseases (bronchiolitis, pneumonia, encephalitis, meningitis, parotitis, orchitis).

The aim of our study was to examine the presence of paramyxovirus and hantavirus genetic material in bats, collected on the Balkan peninsula.

MATERIALS AND METHODS

Ninety-five deceased bats were collected on nine different sites in Serbia and also two sites in the neighboring Republic of Montenegro, both countries in the South-east Europe (Figure 1), in the period 2002 to 2009. Bat carcasses were morphologically identified to species and stored at -20°C until further examination. Nucleic acid isolation was



Figure 1. Map of Serbia showing the locations of the collecting sites, including two sites in Montenegro. In the corner: position of Serbia in Europe and Balkan peninsula.

performed on pooled samples from two consecutive animals. Isolation of total RNA from animal lungs was performed using TRIZOL Reagent (GibcoBRL, Invitrogen, Karlsruhe, Germany) according to manufacturer's instruction. DNA extraction was performed using PureLink Viral DNA Mini Kit (Invitrogen, USA). All samples were tested with universal primers for cytochrome-b gene and for beta-actin mRNA as RT-PCR and PCR positive controls. Viral RNA was detected by nested RT-PCR method, using Thermo Scientific Verso 1-Step RT-PCR ReadyMix kit (Invitrogen, USA) for the outer reaction and Thermo Scientific

DreamTaq Green PCR Master Mix kit (Invitrogen, USA) for the inner PCR reaction. Degenerate primers designed from the highly conserved L segment region of all known hantaviruses and primers that afford amplification of both paramyxovirus subfamilies were employed in our search for hantavirus and paramyxovirus RNA, yielding 390 bp and 200-500bp products, respectively (Klempa *et al.*, 2006; Tong *et al.*, 2008). The list of PCR primers used in the study is shown in Table 1. Negative and positive controls were included in all rounds of RT-PCR. Obtained RT-PCR products were visualized by 1% agarose gel electrophoresis.

Table 1. List of PCR primers used in the study

beta-actin primers	
B1-F	ATG GTN GGN ATG GGN CAR AAR
B1-R	DAT CCA CAT YTG YTG RAA NGT
Cytochrome b primers	
CytB-F	ACT GGY TGD CCB CCR ATT CA
CytB-R	TCA TCM TGA TGA AAY TTY GG
Universal hantavirus L segment primers	
HAN-L-R1	AAC CAD TCW GTY CCR TCA TC
HAN-L-F1	ATG TAY GTB AGT GCW GAT GC
HAN-L-F2	TGC WGA TGC HAC IAA RTG GTC
HAN-L-R2	GCR TCR TCW GAR TGR TGD GCA A
Primers for detection of L gene of Paramyxovirinae	
PAR-F1	GAA GGI TAT TGT CAI AAR NTN TGG AC
PAR-F2	GTT GCT TCA ATG GTT CAR GGN GAY AA
PAR-R	GCT GAA GTT ACI GGI TCI CCD ATR TTN C
Primers for detection of L gene of Pneumovirinae	
PNE-F1	GTC CCA CAA ITT TTG RCA CCA NCC YTC
PNE-F2	ACT GAT CTI AGY AAR TTY AAY CAR GC
PNE-R	GTG TAG GTA GIA TGT TYG CNA TGC ARC C

RESULTS

Collected animals were identified as: *Rhinolophus ferrumequinum*, *Hypsugo savii*, *Myotis alcaethoe*, *Myotis brandtii*, *Myotis myotis*, *Myotis mystacinus*, *Myotis oxygnathus*, *Pipistrellus pipistrellus*, *Pipistrellus pygmaeus*, *Pipistrellus nathusii*, *Plecotus auritus*, *Plecotus austriacus*. All sample pools tested positive for beta-actin mRNA and/or cytochrom B DNA, confirming successful nucleic acid isolation and amplification. However, no sample tested positive for virus specific RNA.

DISCUSSION

Bats represent an important proportion of about 20% of the known terrestrial species of mammals (Kohl & Kurth, 2014). The European continent is inhabited by 52 bat species, whereas 29 bat species from three families (Rhinolophidae, Vespertilionidae and Miniopteridae) have been recorded in Serbia so far (Karapandza & Paunovic, 2008). All 29 bat species identified in Serbia have a status of strictly protected wild species

(Paunovic *et al.*, 2011). Bats have recently been recognized as potential reservoir of emerging human pathogens, including hantaviruses and paramyxoviruses (Hun Gu *et al.*, 2014; Conrardy *et al.*, 2014). Among the multiple factors that facilitate virus evolution within and transmission from bats are their longevity, migratory activity, large and dense roosting communities, and close social interaction (Calisher *et al.*, 2006; Plowright *et al.*, 2015). Given the potentially devastating effects of these emerging diseases on public health and wildlife conservation, it is important to evaluate and survey the presence of diverse viral pathogens in bats.

Hantaviruses are known to be carried by rodents, insectivores and bats and transmitted to humans by inhalation of virus containing aerosols of infected excreta. In humans, they cause two clinical syndromes: hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas, both related to distinct hantaviral causative agents. Increasingly, new hantaviruses are being described and new animal reservoirs discovered (Yanagihara *et al.*, 2014).

Hantaviruses are endemic in the Balkan region, particularly in Serbia, where sporadic cases and/or outbreaks of HFRS have been repeatedly reported. Ever since the first clinical and epidemiological evidence of HFRS in Serbia, dating mid-last century, serological evidence of hantaviral infection has been detected, in both humans and animal reservoirs (Diglišić *et al.*, 1994; Gligić *et al.*, 1989). Positive detection of hantaviral antigens and/or antibodies has been found in multiple species of rodents (*A. flavicollis*, *A. sylvaticus*, *A. agrarius*, *A. microps*, *M. arvalis*, *M. subterraneus*, *M. musculus*, *R. norvegicus*, *My. glareolus*) and insectivores (*S. alpinus*, *S. araneus*, *C. suaveolens*) (Gligić *et al.*, 1992; Gligić, 2008). Moreover, we have recently described hantaviral genetic material in a species of the *Gliridae* rodent family (Stanojević *et al.*, 2015). The evolution of hantaviruses was previously thought to be inextricably linked with the evolution of their hosts, but this relationship has recently been hotly debated. Host switching was demonstrated during the evolution of insectivore-borne hantaviruses but also the evolution of rodent-borne hantaviruses seems not to have strictly followed the coevolution theory (Reusken & Heyman, 2013).

Paramyxoviruses represent a numerous viral family that comprises common and important human pathogens such as measles virus, mumps virus, respiratory syncytial virus. The zoonotic potential of paramyxoviruses is particularly demonstrated by their broad host range. The emergence of Hendra and Nipah virus provided the first evidence of a zoonotic paramyxovirus originating from bat (Drexler *et al.*, 2012b).

No evidence of viral genetic material upon molecular investigation has been described in several studies so far. Negative results from PCR testing of European bats for different human-pathogenic viruses has been reported for flavi-, hanta-, hepadna- and influenza-A viruses, in a few studies analyzing different number of samples, ranging from 42 to 1369 (Drexler *et al.*, 2013; Mühldorfer *et al.*, 2011; Fereidouni *et al.*, 2013). However, a recent RT-PCR screening of bat fecal samples for astro-

calici-, corona-, lyssa-, othoreo-, paramyxo-, and rotaviruses was performed in the neighboring Hungary, yielding positive findings in nearly one in ten tested samples for viral RNA (Kemenesi *et al.*, 2014). Since the study material in our analysis originates from animal carcasses, we assessed the quality by analyzing it using universal primers cytochrome-b gene and for beta-actin gene, in both PCR and RT-PCR reactions, yielding positive signals, however, prudent interpretation is needed. Lack of detection of specific viral RNA may be due to tissue degradation prior to collection. However, positive results in control reactions for PCR and RT-PCR with universal primers imply sufficient integrity of total DNA and RNA for negative finding of viral RNA to be interpreted as true.

We report negative finding of viral genetic material in bats, in the first study exploring bats as paramyxovirus and hantavirus reservoirs in Serbia. In view of challenges in studies involving protected and endangered species, approaches involving better quality and non-invasive sampling are needed. Further investigations are underway to research bats as putative reservoirs of emerging viruses in the Balkans.

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