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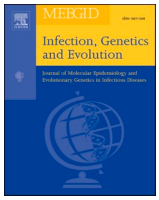

Short communication

Brno loanvirus (BRNV) in bats inhabiting the urban area of Brno, Czech Republic

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ABSTRACT

Bats are known reservoirs of various emerging pathogens, and have recently been found to host a novel hantavirus, named Brno loanvirus (BRNV), from the *Mammantavirinae* subfamily (family *Hantaviridae*, order *Bunyavirales*). Here we report BRNV detection in bats from the urban area of Brno, Czech Republic in March 2022. Specifically, we uncovered a high prevalence of BRNV (8.8%, 5/57) among hibernating bats (*Nyctalus noctula*) in urban area, which poses a risk of human exposure. The positive bats included adult females (3/9 positive), a juvenile female (1/32 positive), and an adult male (1/6 positive). All 10 juvenile males were negative. We used RT-qPCR to quantify the BRNV RNA levels in various bat organs, which yielded positive results for viral RNA in organs, including the kidneys, heart, spleen, brain, liver, lung, and gut, and in body cavity fluid. Among all tested organs, the liver showed the highest levels of viral RNA in 4 out of 5 animals examined (average Ct value of 20.8 ± 7.4).

1. Introduction

Hantaviruses—classified in the family *Hantaviridae*, order *Bunyavirales* (Laenen et al., 2019)—are a diverse group of viruses, some of which are important zoonotic viruses with public health implications. Hantaviruses possess a tri-segmented negative-sense RNA genome, including a small (S) genomic segment, encoding the viral nucleocapsid protein N; medium (M) segment, encoding envelope glycoproteins; and a large (L) segment, encoding the viral RNA-dependent RNA polymerase (Vaheri et al., 2013).

Rodents are the primary natural hosts for hantaviruses, although bats, moles, shrews, reptiles, and fish have also been identified as reservoirs (Witkowski et al., 2014; Avšič-Županc et al., 2019; Laenen et al., 2019). These natural hosts typically harbor persistent infection with minimal biological impact. Notably, rodents excrete hantaviruses in saliva, urine, and faeces, posing a risk of human infection via inhalation or, rarely, rodent bites (Vaheri et al., 2013; Avšič-Županc et al., 2019).

Bats are renowned reservoirs for various viral agents—including filoviruses, rabies virus, and novel coronaviruses (CoVs)—and play a crucial role in public health (Luis et al., 2013; Drexler et al., 2012; Tiwari et al., 2020). Bats host over 130 viral species, of which approximately 60 are zoonotic and exhibit significant pathogenicity in humans. Notable examples include SARS-like coronavirus (SL-CoV), Ebola virus, Nipah virus, and Hendra virus (Luis et al., 2013; Li et al., 2005; Leroy et al., 2005; Rahman et al., 2010; Halpin et al., 2000).

Our research group discovered a novel hantavirus, named Brno loanvirus (BRNV, *Loanvirus brunaense*), in the common noctule bat (*Nyctalus noctula*) in the Czech Republic (Straková et al., 2017). Analysing the complete sequence of all coding genomic regions revealed that BRNV exhibits phylogenetic proximity to other bat-borne hantaviruses, and considerable genetic distance from other known hantaviruses. Expanding on this discovery, Dafalla et al. (2023) investigated BRNV distribution and host specificity among bats in Germany, Austria, and Poland. Their screening confirmed BRNV presence in common noctule bats across different countries. They examined carcasses of 25 other bat species, but only found BRNV in *N. noctula*, suggesting a host-specific relationship. Their study revealed broad distribution of BRNV in Central Europe, with 1.2% prevalence in Germany, 0.5% in Austria, and

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15% in Poland. Furthermore, RT-qPCR analysis of viral RNA distribution highlighted the highest RNA loads in the liver, lung, and kidneys (Dafalla et al., 2023).

In our current study, we investigated the presence of BRNV in a population of common noctules inhabiting urban environments, with specific focus on bats collected from the urban region of Brno city, at the end of their hibernation period. This approach is crucial to assess the potential risk of human exposure to BRNV, considering the presence of common noctules in human settlements.

2. Material and methods

2.1. Bat collection

N. noctula bats were collected from the Faculty of Medicine building at Masaryk University in Brno, Czech Republic. Bats hibernate in this building's ventilation shafts, with over 1000 individuals recorded during the winter of 2021/2022. Following a rapid warm-up in February and early March of 2022, at the end of hibernation period, 57 freshly deceased common noctules were discovered in the building's atrium, and were used for our study. Standard dissection protocols were followed. Tissue samples (lung, liver, spleen, kidney, brain, gut, and heart) and body cavity fluid were collected and stored at −80 °C.

2.2. BRNV RNA detection and sequencing

From each organ, a 10% homogenate was prepared using the Bead Ruptor Elite (OMNI International). From the 10% liver homogenate, total RNA was isolated using the QIAamp Viral RNA Mini Kit (QIAGEN), for screening purposes. Reverse transcription was carried out using the ProtoScript II First Strand cDNA Synthesis kit (New England Biolabs), according to the manufacturer's instructions. The synthesized cDNA was used for BRNV detection, following the nested RT-PCR protocol outlined by Klempa et al. (2006), targeting 412 bp of the L segment. This assay utilized degenerated primers (HAN-L-F1 and HAN-L-R1 for primary PCR; HAN-L-F2 and HAN-L-R2 for nested PCR). PCR products were analysed by electrophoresis on a 2% agarose gel stained with EliDNA PS Green (Elizabeth Pharmacon). PCR products of expected sizes were purified using the Wizard SV Gel and PCR Clean-Up System (Promega), and sent for Sanger sequencing at Eurofins Genomics (Ebersberg, Germany) to confirm BRNV detection.

2.3. Phylogenetic analyses

The dataset for phylogenetic analyses comprised BRNV sequences, along with sequences of other hantaviruses obtained from the GenBank database (https://www.ncbi.nlm.nih.gov). Alignment was conducted and refined using the BioEdit v.7.0.5.3 program (Hall, 1999). Phylogenetic relationships were reconstructed with the maximum likelihood (ML) and Bayesian inference (BI) approaches, implemented in PhyML v.2.4.3 (Guindon and Gascuel, 2003) and MrBayes v.3.2.2 (Huelsenbeck and Ronquist, 2001), respectively. The best-fitting model of evolution (GTR + Γ + I) was determined using SMS: Smart Model Selection software (http://www.atgc-montpellier.fr/sms/; Lefort et al., 2017). ML computations involved non-parametric bootstrap analysis with 1000 replicates. BI was executed with MCMC for 10 million generations, with tree sampling every 100 generations, and the trees were summarized after discarding 25% as burn-in. Final trees were visualized and exported using TreeView v.1.6.6 (Page, 1996), and adjusted in Adobe Illustrator 2020 (Adobe Systems, Inc.).

2.4. Quatification of BRNV RNA in bat organs

For BRNV quantification, 10% homogenates of liver, kidney, brain, heart, gut, and spleen from BRNV RNA-positive *N. noctula* were prepared and analysed using a semiquantitative RT-PCR developed for this study. Primers and a probe were designed targeting the RNA-dependent RNA polymerase of BRNV (GenBank accession number: KX845680.1) using a modified version of Primer3 version 2.3.7, implemented in Geneious Prime® version 2022.0.2 (Biomatters, Inc., Auckland, New Zealand). Total RNA was isolated from the 10% homogenates using the QIAamp viral RNA Mini Kit (QIAGEN). RT-qPCR was conducted using the Luna Probe One-Step RT-qPCR Kit (New England Biolabs) with oligonucleotides RdRp-fw: 5'-TGCTCACCATTCTGATGATGC-3′ and RdRp-rv: 5'-ACATTGCTTTCCATATATCCCCATT-3′, and the probe RdRp-pr: 5'-TTGAGCCTGAAGATGATGGTTCTGCATGGT-3′. The RT-qPCR reactions were conducted in 96-well plates using the LightCycler® 480 System (Roche). The distribution of BRNV RNA among various bat organs was calculated with GraphPad Prism 7.04 (GraphPad Software, Inc.).

3. Results and discussion

The common noctule (*N. noctula*) is an insectivorous bat that is widespread throughout Europe, Asia, and North Africa. During periods of high energy demands (i.e. during gestation and lactation), female bats often travel further north to take advantage of the higher insect biomass production (Hutterer et al., 2005). Lehnert et al. (2018) demonstrated that male bats can also migrate over long distances across Central Europe.

N. noctula bats tested in this study were collected in urban area of Brno city (Czech Republic) at the end of hibernation period. Study sample (57 freshly deceased common noctules) comprised 73.7% juvenile bats ($n = 42$) [56.1% females ($n = 32$) and 17.5% males ($n = 10$)], and 26.3% adult bats ($n = 15$) [15.8% females ($n = 9$) and 10.5% males ($n = 6$)].

Liver samples were screened using RT-PCR assays targeting the L segment of BRNV, yielding the detection of five BRNV RNA-positive samples among 57 examined bats. The positive bats included adult females (3/9 positive), a juvenile female (1/32 positive), and an adult male (1/6 positive). All 10 juvenile males were negative. The PCR products were sequenced to confirm the assay's specificity, and all five positively tested samples were identified as BRNV. Thus we found an 8.8% BRNV RNA detection rate among *N. noctula*. This occurrence was higher than the BRNV prevalence rates reported by Dafalla et al. (2023) in Germany (0.5%) and Austria (1.2%), but was lower than the BRNV prevalence rate detected in Poland (15%), but observed prevalence in Poland might be biased by the low number of samples (3/20 positive). Notably, Dafalla et al. (2023) provide no detailed characteristics regarding the origins of bat carcasses, prohibiting interpretation of these differences in prevalence. Bats form small maternity colonies of usually only a few dozen females, often in tree roosts or in prefabricated buildings. However, for hibernation, they congregate in larger groups—often hundreds or even thousands of bats—and use crevices in caves, church towers, or blocks of flats (Zahn et al., 2000; Ceľuch and Kaňuch, 2005). Therefore, it is likely that virus transmission occurs during contact between individuals just before hibernation.

Phylogenetic analyses of detected BRNV yielded a well-resolved and well-supported tree. The hantaviruses grouped according to their reservoir hosts (Fig. 1), with two hantavirus clusters infecting insectivores, two clusters exclusively found in rodents, and a large group transmitted solely by bats. Although Old World and New World hantaviruses exhibited a strong clustering pattern (reflecting their geographic distribution), exceptions were observed. BRNV formed a clearly defined and highly supported monophyletic lineage within the bat-borne hantavirus group, associated exclusively with *N. noctula*. Longquan virus, Mouyassue virus, and Nova virus were its closest relatives, although distantly related. BRNV sequences exhibited slight differences, which split them into several sublineages (Fig. 1), however, these differences were attributed to intraspecific variability. No correlations were observed with the bat host's tissue, sex, or geographic origin.

For all five positively tested *N. noctule*, the BRNV tissue distribution

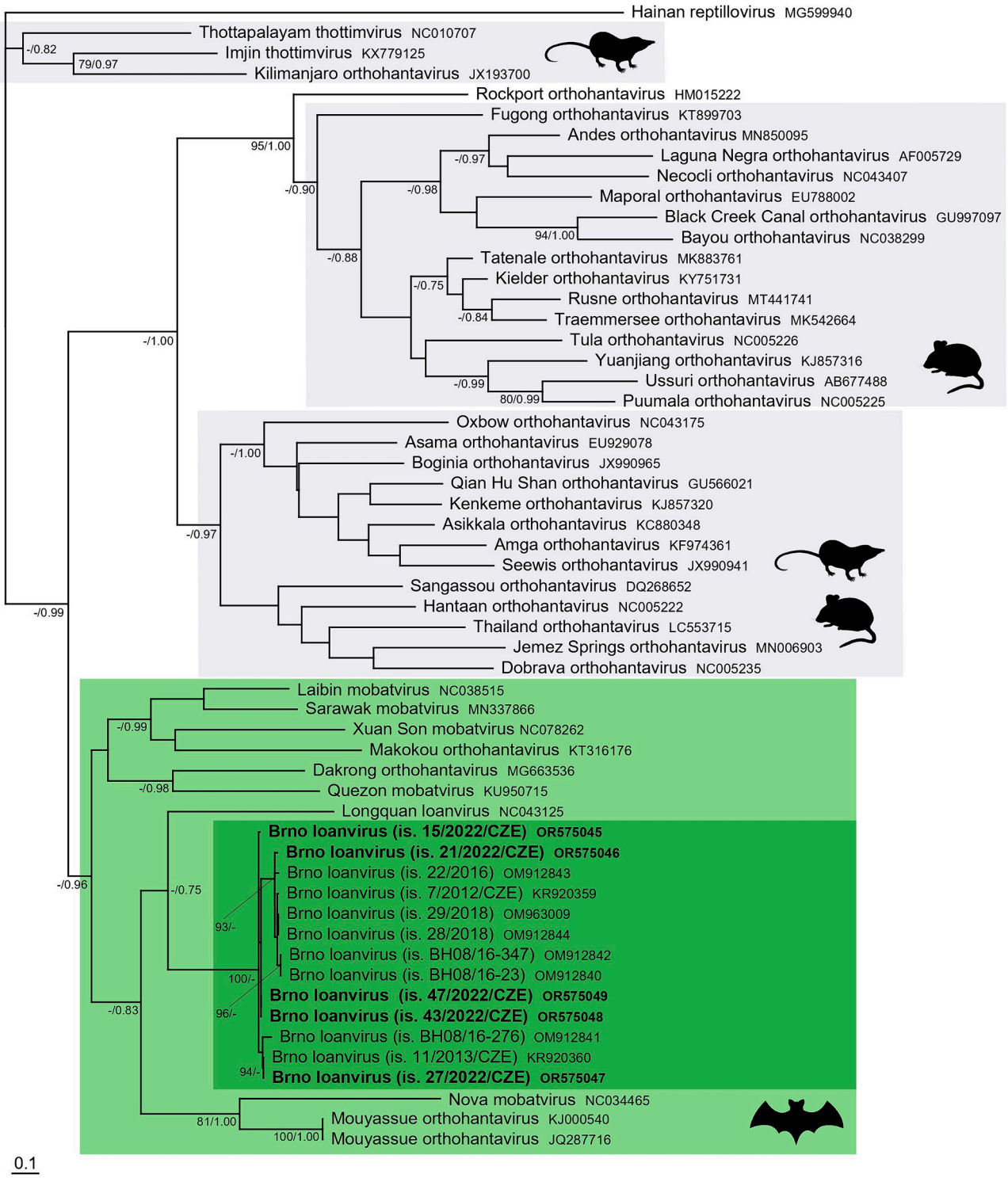

Fig. 1. Phylogenetic relationships of the BRNV loanvirus inferred by the maximum likelihood (ML) analysis of partial sequences of the large (L) segment. The Bayesian inference (BI) tree was mapped on the ML tree. Numbers at the nodes are displayed in order as bootstrap values derived from ML analysis/posterior probabilities under BI analysis. Only bootstrap supports of >70%, and posterior probabilities of >0.70 are shown. If one of the values at the node is lower than 70% or 0.70, it is marked with a hyphen (−). The scale bar indicates evolutionary distance of 0.1 nucleotide substitutions per site. Hainan hantavirus infecting geckos is used as an outgroup. Bold text indicates taxa for which new sequences were obtained in this study.

was examined by semiquantitative RT-PCR. BRNV RNA was detected in all tested organ tissues (Fig. 2), with varying amounts among the tissues. The following average Ct values were detected: kidneys, 30.5 ± 4.3; heart, 32.1 ± 3.5; spleen, 28.8 ± 5.1; brain, 34.4 ± 1.9; liver, 20.8 ± 7.4; lung, 26.3 ± 1.7; gut, 25.3 ± 3.5; and body cavity fluid, 29.9 ± 4.1. The liver contained the highest amount of BRNV RNA, which is in accordance with previous findings of Dafalla et al. (2023).

In summary, here we provide the first demonstration that BRNV exhibits a high prevalence in bats immediately after hibernation. The presence of BRNV in bats from an urban area could pose a potential risk of human exposure; however, the zoonotic potential of this virus remains unknown.

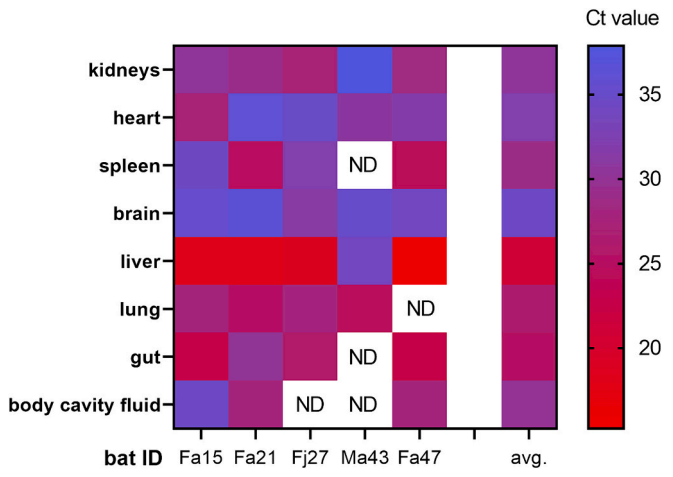

Fig. 2. Heat map showing distribution of BRNV RNA (Ct values) among various organ tissues collected from positively tested *N. noctula*. Bat ID indicates category of animal (Fa: adult female, Fj: juvenile female, and Ma: adult male) and its number in study collection.

Author contributions

A.F.: Molecular detection of BRNV, Wrote the manuscript. **J.S.:** Study conceptualization, Wrote the manuscript. **D.R.:** Study conceptualization, Wrote the manuscript. **T.B.:** Bat collection, Provided input on the manuscript. **P.St:** Bat dissection, Organ homogenate preparation, Provided input on the manuscript. **P.Sv.:** Bat dissection, Organ homogenate preparation, Provided input on the manuscript. **J.H.:** Designed the real-time RT-PCR, Provided input on the manuscript. **J.K.:** Phylogenetic analyses, Provided input on the manuscript.

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CRediT authorship contribution statement

Andrea Fořtová: Investigation, Writing – original draft. **Petra Straková:** Investigation, Writing – review & editing. **Jan Haviernik:** Methodology, Writing – review & editing. **Pavel Svoboda:** Investigation, Writing – review & editing. **Tomáš Bartonička:** Resources, Writing – review & editing. **Jana Kvičerová:** Formal analysis, Writing – review & editing. **Daniel Růžek:** Writing – original draft, Conceptualization, Funding acquisition. **Jiří Salát:** Writing – original draft, Conceptualization, Funding acquisition, Supervision.

Declaration of competing interest

All authors declare no conflict of interests.

Data availability

The data presented in this study can be obtained from the corresponding author upon a reasonable request.

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