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Detection of Brno loanvirus (*Loanvirus brunaense*) in common noctule bats (*Nyctalus noctula*) in Southern Russia

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Abstract

Hantaviruses that infect humans are rodent-derived viruses with zoonotic potential. Several studies show that before emerging in rodents hantaviruses could emerge in bats, which makes it important to study bat-derived hantaviruses. In this study, we performed PCR screening of hantaviruses in samples from common noctules (*Nyctalus noctula* [182 fecal and 81 blood serum samples]), parti-coloured bats (*Vespertilio murinus* [41 fecal samples]), Kuhl's pipistrelles (*Pipistrellus kuhlii* [15 fecal samples]), and serotine bats (*Eptesicus serotinus* [8 fecal samples]) from Rostov Bat Rehabilitation Center (Rostov-on-Don, Russia) and phylogenetic analysis of detected viruses. As a result, hantaviruses were detected in samples from *N. noctula* bats with an overall prevalence of 4.94% (4/81, 95% CI 0.22–9.66%) in blood serum samples and 1.1% (2/182, 95% CI 0–2.61%) in fecal samples. Phylogenetic analysis revealed that detected hantaviruses are highly homologic to Brno loanviruses (*Loanvirus brunaense*) previously discovered in *N. noctula* bats from Central Europe, which brings some evidence that these are the same bat-derived viruses. This study shows that *Loanvirus brunaense* could be species-specific to the host and has a wide area of habitat: from Central Europe to Southern Russia. These are the first findings of this virus in Southern Russia and Ciscaucasus/Fore-Caucasus. Further studies with wider screening and genomic assays of *Loanvirus brunaense* in bats could reveal trends in the molecular evolution of hantaviruses and provide valuable data for the control of potential spillovers.

Keywords Hantaviruses · Bats · Phylogenetics · PCR · Sequencing

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Introduction

Bats are recognized as natural hosts for multiple emerging viruses, such as Hendra, Nipah, Ebola, and Marburg viruses, as well as SARS-CoV, MERS-CoV, and SARS-CoV-2 [1–3]. Unique physiological features, such as limited inflammatory response within antiviral immune reactions, ability to fly, and high longevity make bats ideal "bioreactors" for emerging viruses and their unpredictable spillovers all over the globe [4–6]. This emphasizes the need for constant screening of potential bats-derived zoonotic viruses, especially because the order Chiroptera is the second most diverse mammalian order after Rodentia with more than 1400 species in it and the virome of most of these species is yet to be investigated [7]. Even though bats harbor abundant viral diversity, the vast majority of bat viruses have not emerged to cause disease in other animals and humans [8]. This also highlights the importance of studying bats' viruses, as in combination with the fact that bats relatively peacefully co-exist with viruses due to their unique immune system this data could promote our understanding of viral evolution and its future directions [9, 10].

Hantaviruses, members of the family *Hantaviridae*, pose a huge threat to public health, especially rodent-derived ones belonging to the genus *Orthohantavirus*, as they cause hemorrhagic fever with renal syndrome or hantavirus cardiopulmonary syndrome [11]. Although bats at the moment are not recognized as primary hosts for emerging hantaviruses, the importance of studying these viruses in bats is unconditional, as several phylogenetic studies indicate that hantaviruses might have first appeared in bats before emerging in rodent species [12, 13]. Hantaviruses were previously identified in bats from Africa– Magboi virus in Sierra Leone [14], Mouyassue virus in Cote d'Ivoire [15], Makokou virus in Gabon [16], Kiwira virus in Tanzania and the Democratic Republic of Congo [17]; Asia– Xuan Son virus in Vietnam [18], Huangpi virus, Longquan virus and Laibin virus in China [12, 19], Quezon virus in the Philippines [20], Sarawak mobatvirus in Malaysia [21]; South America - Buritiense virus in Brazil [22]; and Europe– Brno loanvirus (*Loanvirus brunaense*) in Czech Republic, Germany, Austria, and Poland [23, 24]. Interestingly, while most of the bat-derived hantaviruses did not show species specificity, *Loanvirus brunaense* was detected only in common noctule bats (*Nyctalus noctula*) in Straková et al. and Dafalla et al. studies [23, 24]. The common noctule (*N. noctula*) is one of the most common bat species throughout Europe and Asia [25]. This bat species is prone to synanthropy– Printz et al. study showed that common noctules are significantly more abundant in urban areas in comparison to forests [26]. Close contact of common noctules with humans and domestic animals indicates the importance of screening for pathogens in this bat species, as well as in other synanthropic bat species.

This study aimed at screening and phylogenetic characterization of hantaviruses in synanthropic bats from the Rostov Bat Rehabilitation Center at Don State Technical University, Russia.

Materials and methods

Sampling

Sampling was conducted from January to March 2023 at the Rostov Bat Rehabilitation Center (Don State Technical University, Rostov-on-Don, Russia). Bats included in this study belonged to four species: common noctule (*N. noctula*), parti-coloured bat (*Vespertilio murinus*), Kuhl's pipistrelle (*Pipistrellus kuhlii*), and serotine bat (*Eptesicus serotinus*). All bats included in this study were seized by Don State Technical University bat rehabilitation center volunteers starting from November 2022 to March 2023 from households at the request of the owners. Then, seized colonies and separate individuals were transferred to the rehabilitation center, where they underwent veterinary examination and care. Bats of one species were kept collectively (from 5 to 20 individuals) in one box to ensure socialization during rehabilitation with hibernation included. The diet of bats while in the rehabilitation center consisted of superworms and mealworms. Bats were put into controlled hibernation in late November 2022 at a constant temperature of 10 °C and brought out in early April 2023. A minimum of 0.5 g of fecal samples were collected from boxes with hibernating bats: 182 samples from *N. noctula*, 41 samples from *V. murinus*, 15 samples from *P. kuhlii*, and 8 samples from *E. serotinus*. Sampling was conducted once from each box. Obtained fecal samples were stored at − 80 °C. Also, for the study, a minimum of 50 µL of blood samples were collected from 81 male *N. noctula* bats. Blood samples were collected by venipuncture of the brachial vein according to the Smith et al. protocol [27]. Blood sampling from female individuals and certain other bat species was not performed due to ethical concerns. For instance, venipuncture in female bats poses a risk for potentially pregnant individuals (there were only female *E. serotinus* in the rehabilitation center at the time of sampling), and the smaller body size of species like *V. murinus* and *P. kuhlii* made it difficult to collect a sufficient amount of blood safely. After blood sampling, plasma specimens were collected by centrifugation (1000 g, 15 min) and stored at -80 °C. All animals included in this study were clinically healthy according to the basic veterinarian examination and showed no signs of infections.

Hantaviruses detection: RNA extraction, PCR, and sequencing

For viral RNA extraction, 50 mg of each fecal sample was homogenized in 500 µL of PBS with vortexing until a homogeneous suspension was obtained. Then after centrifuging with 11,000 rpm for 4 min, supernatant was obtained, 100 µL of which was used for further RNA extraction. Viral RNA was extracted from blood serum and homogenized fecal supernatant samples using the RIBO-prep kit (AmpliSens, Moscow, Russia). Reverse transcription was performed using the REVERTA-L kit (AmpliSens, Moscow, Russia).

For PCR screening to detect hantaviruses, we used two pairs of primers for L-segments of hantaviruses from the Klempa et al. study: Han-L-F1 (5′-ATGTAYGTBAGTGCWGATGC-3′) and Han-L-R1 (5′-AACCADTCWGTYCCRTCATC-3′); Han-L-F2 (5′-TGCWGATGCHACI-AARTGGTC-3′) and Han-L-R2 (5′- GCRTCRTCWGARTGRTGDGCAA −3′) [28]. The synthesis of oligonucleotides, including degenerate primers, was carried out by Evrogen (Moscow, Russia). PCR was conducted using PCR 5X ScreenMix (Evrogen, Moscow, Russia) and T100 Thermal Cycler (BioRad, Hercules, California, USA) with the following thermal cycles for the first pair of primers– 1 cycle of 5 min at 93 °C, 34 cycles of 30 s at 93 °C, 20 s at 55 °C, 30 s at 70 °C, 1 cycle of 5 min at 70 °C and 4 °C at the end of the reaction, and for the second pair of primers– 1 cycle of 5 min at 93 °C, 34 cycles of 30 s at 93 °C, 20 s at 59 °C, 25 s at 70 °C, 1 cycle of 5 min at 70 °C and 4 °C at the end of the reaction. Detection of amplification products was performed in 1.5% agarose gel by horizontal electrophoresis using Gel Doc XR + Gel Documentation System (BioRad, Hercules, California, USA). The prevalence of positive signals was calculated as percentages with 95% confidence intervals (95% CI). Extraction and purification of amplification products from agarose gel were conducted using the N-Gel kit (Biolabmix, Novosibirsk, Russia). Sequencing of obtained amplicons was carried out with SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Nucleic and amino acids sequence identity analysis

Identity analyses of the sequenced nucleic acids of L-segments of hantaviruses and corresponding amino acid sequences were conducted using MEGA software (version 11) [29] and previously published L-segment sequences of hantaviruses from GenBank (NCBI). The length of analyzed fragments was 313 bp and 105 amino acids.

Phylogenetic analysis of detected hantaviruses

Detected and sequenced hantavirus L-segments were used for phylogenetic analysis. The majority of sequences for the phylogenetic analysis were obtained from previously published studies [22–24]. Additionally, 10 more sequences of hantaviruses previously associated with human infections were obtained and included 2 samples of each enlisted: *Orthohantavirus seoulense* [30, 31], *Orthohantavirus tulaense* [32, 33], *Orthohantavirus dobravaense* [34], *Hantaan orthohantavirus* [35], and *Orthohantavirus sinnombreense* [36]. All the data was downloaded from the NCBI GenBank [37] database using the Entrez Direct (version 21.6) package [38]. The dataset included both partial and complete coding sequences (CDS) of the L-segment. A total of 103 sequences were included in the analysis. Multiple sequence alignment was performed using the MAFFT (version 7.525) program [39]. To find the best substitution model ModelFinder (implemented in IQ-TREE2) was used [40]. Phylogenetic tree construction was carried out using IQ-TREE2 (version 2.3.0) [41] under the GTR + F + I + G4 substitution model. Branch support was assessed with 1000 ultrafast bootstrap replicates [42]. The resulting phylogenetic tree was visualized using the Interactive Tree Of Life (iTOL, version 6) software [43].

After conducting phylogenetic analysis, a geographic representation of the spatial distribution of detected bat-derived hantaviruses from Rostov-on-Don in this study and closely related viruses described in previous studies was created. The map was constructed utilizing the rnaturalearth package (version 1.0.1) for data sourcing [44] and the ggplot2 package for visualization [45].

The complete bioinformatic pipeline for the phylogenetic analysis and the creation of a spatial distribution map of studied hantaviruses is provided in the Data Availability Statement.

Results

Positive signals on the detection of RNA of hantaviruses were found in 4 of 81 blood serum samples (4.94%, 95% CI 0.22–9.66%) and 2 of 182 fecal samples (1.1%, 95% CI 0–2.61%) from *N. noctula* bats. There were no positive signals in samples acquired from other bat species included in this study.

We acquired two unique L-segment sequences of the detected hantaviruses. Five out of the six sequenced L-segments were exact duplicates. The sequence submitted to GenBank under accession number OR934973 was obtained from three blood serum samples and two fecal samples. The second unique sequence, submitted under accession number

OR934974, was derived from a blood serum sample of a male *N. noctula* bat. According to the results of the phylogenetic analysis of detected hantaviruses based on L-segments (Fig. 1), they appeared to be in one clade with *Loanvirus brunaense* detected in *N. noctula* bats from the Czech Republic, Germany, Austria, and Poland (Fig. 2). Nucleic and amino acid sequences of L-segments of detected hantaviruses were 93.6–100% identical to Brno loanviruses (Table S1).

Discussion

In this study, we conducted a screening of hantaviruses in bats from Rostov Bat Rehabilitation Center and a phylogenetic analysis of detected viruses based on sequenced L-segments. Bat rehabilitation centers provide unique opportunities for studying the microbiota of these animals, which, along with rehabilitation itself, meets the goals of bats' conservation, as information about their microbiota is crucial for bats' health management and anti-epizootic measures to prevent potential spillovers of emerging pathogens

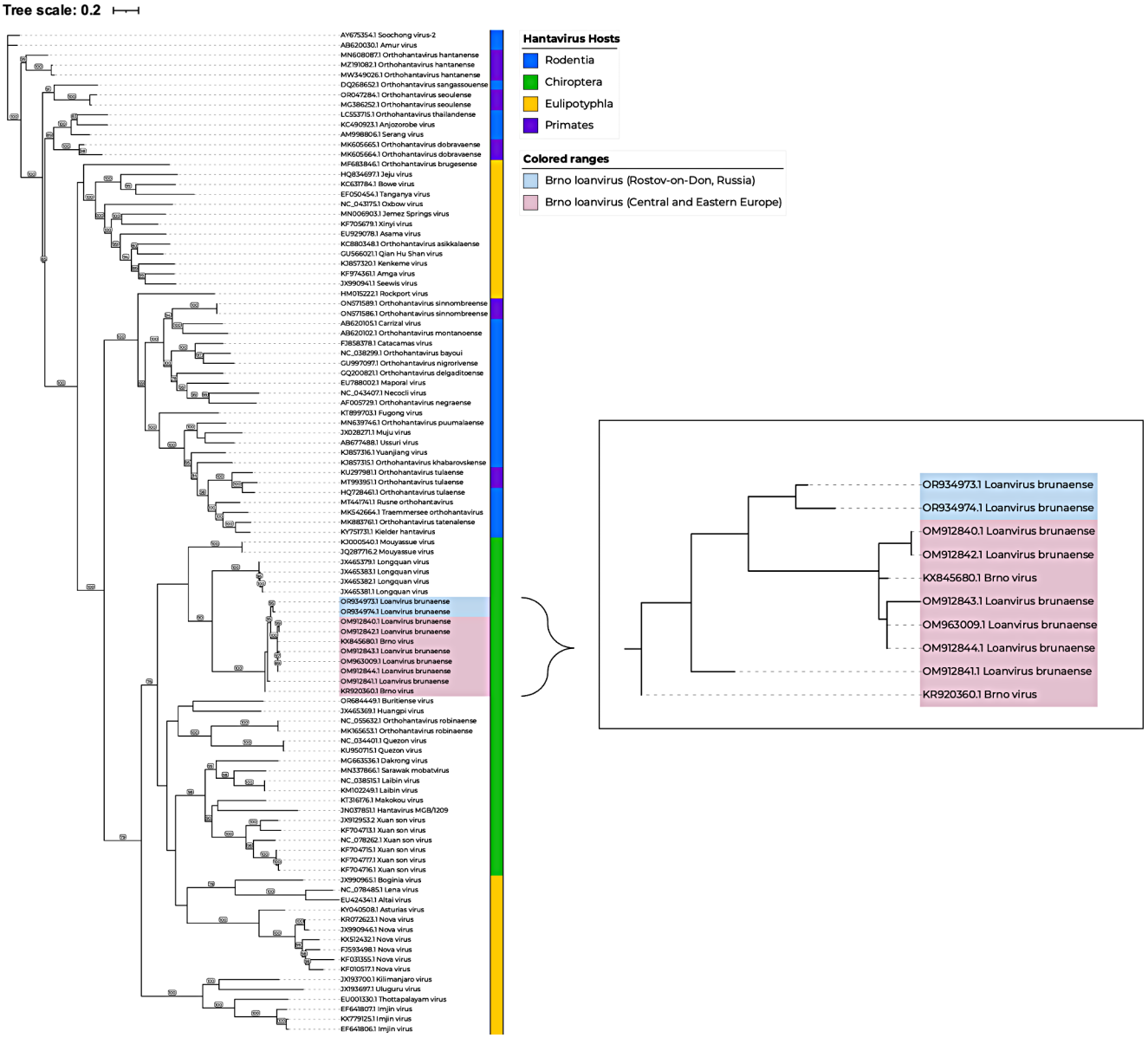

Fig. 1 Maximum likelihood phylogenetic tree constructed using IQ-TREE2 based on complete and partial L segment nucleotide sequences (353 nt) of hantaviruses. Bootstrap values higher than 67 are displayed. The light blue color range indicates Brno loanviruses (*Loanvirus brunaense*) detected in Rostov-on-Don, and the pink range indicates Brno loanviruses detected in Central Europe in previous studies. Analyzed viral sequences were also grouped according to the order of the host: blue– Rodentia, green– Chiroptera, solid yellow– Eulipotyphla, and purple– Primates (*Homo sapiens* only)

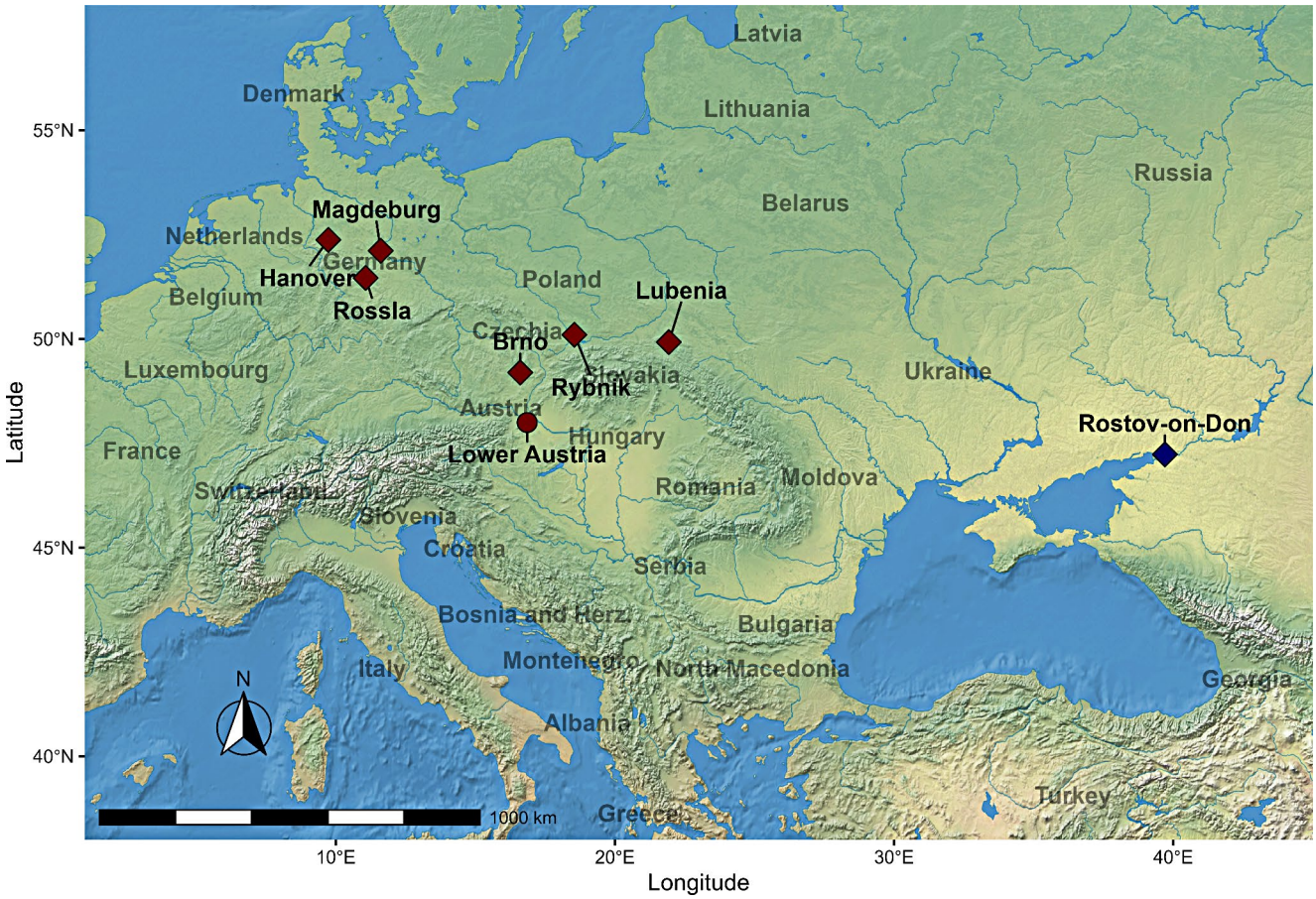

Fig. 2 Locations, where Brno loanviruses (*Loanvirus brunaense*) were detected: Czechia (Brno) [23], Germany (Hanover, Rossla, Magdeburg), Austria (Lower Austria), Poland (Rybnik, Lubenia) [24], and Russia (Rostov-on-Don, this study)

[46–48]. Bats included in our study are prone to synanthropy and were initially found by citizens of Rostov-on-Don in their households with the following seizing and transportation to the rehabilitation center where they were put into controlled hibernation. Close contact with humans additionally highlights the importance of screening for potential pathogens in bats [49–51].

According to the results of PCR screening, hantaviruses were detected only in samples from *N. noctula*. We should note that fecal samples were taken from hibernating bats, which could altered the abundance of viruses. Previous studies showed that the abundance of some bacteria and overall microbial diversity was lower in the gut microbiota of hibernating bats, *N. noctula* in particular [52–54]. However, this could not be true related to the viral abundance and diversity and needs to be tested in future studies.

Phylogenetic and nucleic and amino acids sequence identity analyses of L-segment sequences revealed that detected hantaviruses are highly homologic to *Loanvirus brunaense*, which brings some evidence that these are the same bat-derived viruses. Brno loanvirus (*Loanvirus brunaense*) was initially identified in two liver and one kidney samples collected in 2012 and 2013 from common noctules in Brno (Czech Republic) by Straková et al. [23]. A recent study of the hantaviruses' prevalence in bat populations of Central Europe also detected *Loanvirus brunaense* in tissue samples of common noctules: the hantavirus RNA detection rate ranged between 3 of 20 (15%, 95% CI 0–30.65%) for Poland, 3 of 245 (1.2%, 95% CI 0–2.6%) for Germany and 1 of 207 (0.5%, 95% CI 0–1.43%) for Austria [24]. Interestingly, along with the *N. noctula*, these studies included 24 other bat species of bats, and *Loanvirus brunaense* was identified only in *N. noctula* bats [23, 24], as well as in our study, which included samples from 4 different bat species. These show that *Loanvirus brunaense* could be species-specific to the host. Although *Loanvirus brunaense* has been identified in Central and Eastern Europe, we speculate that this virus could be detected in other regions of the common noctule's habitat. *N. noctula* is a partial and differential migrant bat species capable of overcoming distances up to 1,600 km between hibernation areas [55]. The area of geographical distribution of this bat species lies throughout Eurasia, from the British Isles to Southern Asia, where subspecies of this bat are presented, for example, *N. n. labiatus* [25]. Given

the geographical widespread and migration potential of this bat species, there are several concerns about the emerging potential of detected *Loanvirus brunaense*, which could be put to an end by conducting multiple in vitro and in vivo tests. However, we should mention that according to the phylogenetic analysis detected viruses were not closely related to hantaviruses that cause infections in humans, which is also consistent with previous studies [23, 24].

There were several studies on hantavirus surveillance conducted in Russia. Tkachenko et al. analyzed the public health data on the occurrence of hemorrhagic fever with renal syndrome in Russia collected from 2000 to 2022 and found that there were 4.9 cases per 100,000 population. This incidence rate was not equal in different regions: European Russia accounted for 98.5% of these cases, with an incidence rate of 9.7 per 100,000, while Asian Russia reported 1.5% of cases, with a rate of 0.6 per 100,000. Among the causative agents of these infections, there were six rodent-derived hantaviruses, and 97.7% of hemorrhagic fever with renal syndrome cases were caused by *Puumala orthohantavirus*, the natural reservoir of which is the bank vole (*Myodes glareolus*) [56]. Yashina et al. discovered a novel virus belonging to *Orthohantavirus* genus– Academ Virus in samples 14 out of 18 collected from the Siberian mole (*Talpa altaica*) between 2017 and 2021. Phylogenetic analysis revealed that this virus shared a common evolutionary origin with the Bruges virus (*Orthohantavirus brugesense*), previously identified in the European mole (*Talpa europaea*), and is distantly related to other mole-borne hantaviruses [57, 58]. In addition to these findings, Yashina et al. examined the distribution and phylogeny of the Seewis virus (*Orthohantavirus seewisense*) and Altai virus, belonging to the *Orthohantavirus* genus, in Asian Russia. Both viruses co-circulated in shrew populations in Western Siberia, with the Lena River virus (*Mobatvirus lenaense*) and an *Orthohantavirus seewisense* variant co-circulating in Eastern Siberia. These findings suggest Altai virus and related viruses emerged from ancient cross-species transmission among Sorex shrews in Eurasia [59]. Overall, most of the studies of hantaviruses in wild animals conducted earlier in Russia revealed the wide diversity of rodent- and shrew-derived hantaviruses from *Orthohantavirus* and *Mobatvirus* genera. Bat-derived hantaviruses detected in our study were distinct from these genera and appeared in one clade within the *Loanvirus* genus, which expands the current data on hantavirus diversity in small mammals of Russia.

A limitation of this study is that serum samples from male *N. noctula* were used for hantavirus screening due to ethical restrictions on conducting research in a rehabilitation center. Nevertheless, the overall *Loanvirus brunaense* prevalence rate in common noctules in this study generally corresponds to previous epizootic reports [23, 24], indicating that *Loanvirus brunaense* prevalence may not be related to the sex of the common noctule. Also, the next studies on hantavirus surveillance in bats in Southern Russia should include inner organ samples in addition to blood serum and fecal samples, as in some previous studies, hantaviruses were detected in tissue samples of bats [17, 60]. In addition, further research should be aimed at full-genomic assay of *Loanvirus brunaense* detected in Rostov-on-Don, as this data will provide valuable information regarding the molecular evolution of hantaviruses in bats and could support the evidence that hantaviruses initially appeared in bats and then were transmitted to rodents [61, 62]. Nonetheless, our screening study by itself without full-genomic assessment provides valuable insights into the presence of *Loanvirus brunaense* and its prevalence in the common noctules' population in Rostov-on-Don, which reveals the greater geographical distribution of this virus.

Conclusion

In this study, we discovered Brno loanvirus (*Loanvirus brunaense*) in *Nyctalus noctula* bats from Rostov Bat Rehabilitation Center (Rostov-on-Don, Russia) with an overall prevalence of 4.94% (4/81, 95% CI 0.22–9.66%) in blood serum samples and 1.1% (2/182, 95% CI 0–2.61%) in fecal samples. These are the first findings of this virus in Southern Russia and Ciscaucasus/Fore-Caucasus. We did not detect *Loanvirus brunaense* in samples from *Vespertilio murinus*, *Pipistrellus kuhlii*, and *Eptesicus serotinus*, which shows that this virus could be species-specific to the host. These results are consistent with previous studies, where *Loanvirus brunaense* was detected in *N. noctula* bats in the Czech Republic, Germany, Austria, and Poland. This study shows that *Loanvirus brunaense* has a wide area of habitat: from Central Europe to Southern Russia, which borders Asia, and probably it is not limited to these regions, as *N. noctula* bats' geographical distribution lies throughout Eurasia. Further studies with wider screening and genomic assays of *Loanvirus brunaense* in bats could reveal trends in the molecular evolution of hantaviruses and provide valuable data for the control of potential spillovers.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42770-024-01587-5.

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Data availability Detected Brno loanviruses (*Loanvirus brunaense*) L-segment sequences have been deposited in the GenBank under accession numbers OR934973 and OR934974. The bioinformatic pipeline used for the data analysis has been deposited in GitHub: https://github.com/PopovIILab/PhoBl.

Declarations

Conflict of interest The authors declare no competing interests.

Institutional review board statement The reported study does not involve the results of any in vivo intervention experiments. Handling of animals at the bat rehabilitation center and sampling were approved by the local ethics committee of Don State Technical University (Protocol No. 5 2022).

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