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Short Report

Failure to Detect Hantavirus in Vesper Bats in Poland

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Abstract

Objective: We previously reported the co-circulation of genetically distinct hantaviruses (family *Hantaviridae*) in shrews and moles (order Eulipotyphla, families Soricidae and Talpidae) in central and southeastern Poland. In this exploratory study, we attempted to detect hantavirus in patagia of vesper bats (order Chiroptera, family Vespertilionidae) from Poland.

Methods: RNAlater®-preserved patagia and feces from 88 vesper bats, collected during 2013, 2014 and 2017 in northern, central and south-central Poland, were analyzed for hantavirus RNA by nested reverse transcription polymerase chain reaction (RT-PCR).

Results and Discussion: Despite repeated and exhaustive attempts, using oligonucleotide primers and PCR cycling conditions that led to the discovery of bat-borne hantaviruses in Asia and Africa, we failed to detect hantavirus RNA in vesper bats from Poland, including the common noctule (*Nyctalus noctula*), which has recently been shown to harbor a hantavirus in the Czech Republic. More extensive studies of visceral organs from bats in Poland and elsewhere in Europe are warranted to ascertain the genetic diversity and phylogeography of bat-borne hantaviruses.

Keywords: Hantaviridae; Vesper Bat; Chiroptera; Poland; RT-PCR

Introduction

The discovery of genetically distinct hantaviruses (family *Hantaviridae*) in multiple species of shrews and moles (order Eulipotyphla, families Soricidae and Talpidae) from widely separated geographic regions in Europe, Asia, Africa and/or North America has thoroughly upended the conventional view that rodents are the principal reservoir hosts [1,2]. Earlier, we reported Seewis virus in the Eurasian common shrew (*Sorex araneus*) in Switzerland [3], Finland [4] and Hungary [4]. Subsequently, we reported the co-circulation of Seewis virus in the Eurasian common

shrew and Eurasian pygmy shrew (*Sorex minutus*), Boginia virus in the Eurasian water shrew (*Neomys fodiens*), and Nova virus in the European mole (*Talpa europaea*) in central and southeastern Poland [5,6]. Seewis virus has also been detected in Eurasian common shrews in Germany [7,8], Slovakia [7] and Slovenia [9]; and Asikkala virus has been reported in the Eurasian pygmy shrew in the Czech Republic and Germany [10].

In addition to shrews and moles, bats (order Chiroptera) are now known to harbor highly divergent lineages of hantaviruses [11]. The analysis of more than 3,000 frozen, RNAlater® preserved and ethanol-fixed tissues from bats representing 11 families by reverse transcription polymerase chain reaction (RT-PCR) has yielded 13 hantaviruses to date in bat species belonging to the suborders Yinpterochiroptera (families Hipposideridae, Pteropodidae and Rhinolophidae) and Yangochiroptera (families Emballonuridae, Nycteridae and Vespertilionidae). Of the 13 bat-borne hantaviruses, seven are from Asia: Xuân Sơn virus and Đakrông virus in Vietnam [12-15], Láibīn virus in China and Myanmar [14,16], Huángpí virus and Lóngquán virus in China [17], Sarawak virus in Malaysia [18], and Quezon virus in the Philippines [19]; and five are from Africa: Mouyassué virus in Côte d'Ivoire and Ethiopia [20,21], Ponan virus in Côte d'Ivoire [unpublished observations], Kiwira virus in Tanzania [unpublished observations], Magboi virus in Sierra Leone [22], and Makokou virus in Gabon [23]. Thus far, Brno virus, detected in the common noctule (*Nyctalus noctula*) in the Czech Republic [24], represents the only bat-borne hantavirus reported in Europe. Because Brno virus, Huángpí virus, Ponan virus, Mouyassué virus and Sarawak virus have all been detected in bats belonging to the family Vespertilionidae, we analyzed archival specimens from a convenience sample of vesper bats from Poland for hantavirus RNA. In addition, because population genetics studies of bats commonly rely on nonlethal sampling of patagia biopsies stored in ethanol [25-28], we decided to ascertain if patagia would prove useful in screening for hantavirus infection in bats.

Materials and Methods

Specimens

Patagia, or the wing membranes or skin folds between the forelimbs and hind limbs, from 88 vesper bats, representing seven genera and 11 species (Table 1) and designated in the least concern category of the International Union for Conservation of Nature (IUCN) Red List, were studied. Bats were either captured using mist nets or found dead, during 2013, 2014 and 2017 (Table 1). The majority of bats were from Szachownica Cave (Kraków-Wieluń Upland), which is one of the four largest wintering shelters for bats in Poland [29]. Among the 88 bats were four dead common noctules, which were collected during August 2014 at a wind farm in Wicko, a village in the Pomeranian Voivodeship in northern Poland, approximately 16 km northwest of Lębork and 74 km northwest of the regional capital Gdańsk. Live-caught bats were released after obtaining punch biopsies of patagia and feces, which were preserved in RNAlater®.

Bat Species	Collection Site*	Collection Date	Number
Barbastella barbastellus	Szachownica Cave	Oct 2017	20
Eptesicus serotinus	Kurowice	Aug 2017	7
	Łódź	Aug 2017	1
Myotis brandtii	Wicko	Jun 2014	1
	Kurowice	Aug 2017	2
Myotis myotis	Szachownica Cave	Oct 2017	4
Myotis mystacinus	Szachownica Cave	Oct 2017	1
Myotis nattereri	Szachownica Cave	Oct 2017	21
Nyctalus noctula	Wicko	Oct 2017	1
	Wicko	Aug 2014	4
Pipistrellus nathusii	Wicko	Aug 2013	1
Pipistrellus pipistrellus	Wicko	Aug 2014	1
	Wicko	Jul 2014	1
Plecotus auritus	Brzeziny	May 2017	3
	Kurowice	Aug 2017	12
	Szachownica Cave	Oct 2017	6
Vespertilio murinus	Wicko	Aug 2014	1
	Łódź	May 2017	1

Table1: Collection sites and number of vesper bats tested for hantavirus RNA.

*Collection site locations: Wicko: N 54°40'18.13, E 17°47'33.99; Brzeziny: N 51°47'48.50, E 19°45'27.62; Kurowice: N 51°39'53.38, E 19°42'12.33; Szachownica Cave: N 51°03'15.57, E 18°48'26.94

RNA extraction, cDNA synthesis and RT-PCR amplification

RNAlater®-preserved patagia and feces from 88 vesper bats were analyzed for hantavirus RNA by nested RT-PCR, using previously described protocols [3-6, 13-15]. Total RNA was extracted, using the MagDEA RNA100 Kit (Precision System Science, Matstudo, Japan), and cDNA was synthesized, using the PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Inc., Otsu, Japan) with oligonucleotide primer (OSM55F, 5'–TAGTAGTAGACTCC–3'), designed from conserved 5'–end of the S-, M- and L- segments of hantaviruses [30]. First- and second-round PCR were performed in 20-µL reaction mixtures, containing 250 µM dNTP, 2.5 mM $MgCl_2$, 1 U Takara LA Taq polymerase Host Start version (Takara Bio, Inc.) and 0.25 µM of each primer [31]. Initial denaturation at 94 °C for 2 min was followed by two cycles each of denaturation at 94 °C for 30 s, two-degree step-down annealing from 46 °C to 38 °C for 40 s, and elongation at 72 °C for 1 min, then 30 cycles of denaturation at 94 °C for 30 s, annealing at 42 °C for 40 s, and elongation at 72 °C for 1 min, in a Veriti thermalcycler(Applied Biosystems, Foster City, CA, USA) [19, 27].

Results and Discussion

Bats comprise a quarter of the mammalian fauna in Poland, and all but two of the 22 bat species in Poland are members of the family Vespertilionidae, which is among the most speciose of insectivorous bats. Albeit in limited numbers, our study examined patagia from 11 of the 20 vesper bat species in Poland. While the failure to detect hantavirus RNA in patagia of vesper bats was disappointing, we believe the publication of such unsuccessful efforts is important to record in the scientific literature. Moreover, even in published reports of bat-borne hantaviruses, showing comparatively low success rates (Table 2), hantavirus RNA was not detected in the vast majority of bat species tested.

Small sample sizes, poor tissue preservation and degraded RNA probably contributed to our failed efforts. In addition, technical issues, such as oligonucleotide primer mismatches and suboptimal PCR cycling conditions, were likely contributing factors. That said, among the protocols we used was one reported earlier by Klempa and colleagues [32], which has been used successfully by several groups in search of bat-borne hantaviruses.

Brno virus, detected in two of 12 common noctules from the South Moravian Region of the Czech Republic, represents the only bat-borne hantavirus reported to date in Europe [24]. Patagia, and not visceral tissues, from only four common noctules were tested in this study. Thus, apart from the small sample size, patagia and feces may not be suitable to truly assess hantavirus infection in bats. Moreover, hantavirus infection is typically focal and not uniformly distributed within a given host species in time and space. So, it could be that the common noctule population in the particular collection site in Wicko is not infected. The other shortcoming is that the common noctules in this study were found dead and only RNAlater®-preserved patagia were available for testing. Although hantavirus RNA has been detected in ethanol-fixed liver tissues

Virus Name	Host Species	Country	RNA Positive	Reference
Brno	*Nyctalus noctula*	Czech Republic	2/12	24
Đakrông	*Aselliscus stoliczkanus*	Vietnam	1/2	15
Huángpí	*Pipistrellus abramus*	China	1/5	17
Láibīn	*Taphozous melanopogon*	China	1/32	16
	Taphozous melanopogon	Myanmar	2/13	14
Lóngquán	*Rhinolophus monoceros*	China	1/4	17
	Rhinolophus affinis	China	6/26	17
	Rhinolophus sinicus	China	3/135	17
Magboi	*Nycteris hispida*	Sierra Leone	1/18	22
Makokou	*Hipposideros ruber*	Gabon	1/123	23
Mouyassué	*Neoromicia nanus*	Côte d'Ivoire	2/12	20
	Neoromicia capensis	Ethiopia	1/25	21
Quezon	*Rousettus amplexicaudatus*	Philippines	1/15	19
Sarawak	*Murina aenea*	Malaysia	2/2	18
Xuân Sơn	*Hipposideros pomona*	Vietnam	1/5	12
	Hipposideros pomona	Vietnam	5/44	13
	Hipposideros cineraceus	Vietnam	3/6	14

Table 2: Published reports of bat-borne hantaviruses.*

*Two additional bat-borne hantaviruses, Ponan virus and Kiwira virus, are not included in the table because data are still unpublished.

of two banana pipistrelles (*Neoromicia nanus*) from Côte d'Ivoire [20], future studies must focus on testing optimally preserved viscera from newly collected common noctules and other bat species from multiple other trap sites in Poland, such as the World War II era bunker systems which now serve as important hibernation quarters for multiple bat species.

Conclusions

Insights into the evolutionary history and phylogeography of hantaviruses have been forever changed by the discovery of genetically distinct hantaviruses in shrews, moles and bats [1,2]. Based on in-depth phylogenetic analysis, bats may have served as the primordial reservoirs of ancestral hantaviruses [11]. Thus, many more bat-borne hantaviruses probably await discovery, and the failure to detect hantavirus RNA in patagia of vesper bats in Poland is merely an inconvenient setback.

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Conflict of Interest

The authors do not declare any conflicts of interest.

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