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Hantaviruses are emerging RNA viruses that cause human diseases predominantly in Asia, Europe, and the Americas. Besides rodents, insectivores and bats serve as hantavirus reservoirs. We report the detection and genome characterization of a novel bat-borne hantavirus isolated from insectivorous common noctule bat. The newfound virus was tentatively named as Brno virus.

1. Introduction

Hantaviruses (genus *Hantavirus*, family Bunyaviridae) are responsible for life-threatening human diseases: hantavirus cardiopulmonary syndrome (HCPS) in the Americas and hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe (Krüger et al., 2011). Rodents are natural reservoirs of hantaviruses; however, recent studies have demonstrated that insectivores and bats also represent hosts for divergent hantaviruses (Xu et al., 2015; Zhang, 2014; Witkowski et al., 2016). Bats are considered the natural reservoir of a large variety of zoonotic viruses causing serious human diseases, such as lyssaviruses, henipaviruses, severe acute respiratory syndrome coronavirus, and Ebola virus (Li et al., 2010). Genetically divergent bat-borne hantaviruses have been identified in Africa – Moussaviruses (MOYV) in Cote d’Ivoire (Sumibcay et al., 2012), Magboi virus (MGBV) in Sierra Leone (Weiss et al., 2012), and Makokou virus (MAKV) in Gabon (Witkowski et al., 2016) and in Asia – Xuan Son virus (XSV) in Vietnam (Arai et al., 2013), Huangpi virus (HUPV), Longquan virus (LQUV), Laibin virus (LBV) in China (Xu et al., 2015; Guo et al., 2013), and Quezon virus (QZNV) in the Philippines (Arai et al., 2016). Here we report the detection of a novel hantavirus, tentatively named Brno virus (BRNV), in the European insectivorous bat species *Nyctalus noctula* collected in the Czech Republic, Central Europe. For genetic characterization, the three genome segments were sequenced by high-throughput sequencing (HTS).

2. The study

A total of 53 bats were collected during the years 2008–2013 in the South Moravia region in the Czech Republic. The sample collection contained bats that died accidentally or were found dead in the field. Bats represented 14 different species from two families: *Eptesicus nilssonii* (n = 1), *E. serotinus* (n = 1), *Hypsugo savii* (n = 4), *Myotis bechsteinii* (n = 1), *M. daubentoni* (n = 3), *M. emarginatus* (n = 1), *M. mystacinus* (n = 1), *M. myotis* (n = 1), *Nyctalus noctula* (n = 12), *Pipistrellus pipistrellus* (n = 15), *Plecotus auritus* (n = 2), *Pl. austriacus* (n = 2), *Vespertilio murinus* (n = 6) of the family Vespertilionidae and *Rhinolophus hipposideros* (n = 3) of the family Rhinolophidae.

Total RNA was extracted from the lungs, kidneys and livers of all animals using QIAamp viral RNA Mini Kit (QIAGEN) or QIAZOL/TRIZOL method and screened for the presence of hantavirus RNA by a broad-spectrum RT-PCR targeting the large (L) genome segment of hantaviruses (Klempa et al., 2006). Hantavirus RNA was identified in two noctule bats (*N. noctula* – the species was determined morphologically...
and confirmed by HTS-based results of three host genes) collected in the city of Brno. The obtained sequences (369 base pairs, bp) were designated as Brno 7/2012/CZE (amplified from liver tissue) and Brno 11/2013/CZE (identical sequences were obtained from liver and kidney samples) and deposited in GenBank (accession numbers KR920359 and KR920360, respectively).

Attempts to isolate the virus were done in Vero cells and in suckling mice, but were negative.

In order to determine the whole genome sequence of BRNV, IonTorrent HTS was conducted. The sample Brno 7/2012/CZE was selected as most suitable for sequencing based on high viral loads in a novel mice, but were negative.

CZE (identical sequences were obtained from liver and kidney samples) in the library preparation (Gene Read Library Prep Kit, Qiagen). The library was purified and size selected using Ampure XP Beads (Beckman Coulter). Optimal size distribution and quality of the resulting library were verified on a Bioanalyzer in combination with a DNA High Sensitivity Chip (Agilent). The library was quantified with the Ion Library Taqman Quantitation Kit (ThermoFisher). Sequencing was performed on an Ion Personal Genome Machine (ThermoFisher) according to manufacturer's guidelines.

A metagenomics analysis of the complete dataset was conducted using Riemsoft software (Scheuch et al., 2015). Reads classified as related to the family Bunyaviridae were extracted and de-novo assembled into contigs using 454 Sequencing System Software (version 3.0). Resulting contigs were subsequently analyzed using the BioEdit software 7.2.5 (Hall, 1999) and Geneious 9.0.5 (Kearse et al., 2012). The lengths of the sequences of S, M and L gene segments were 1441, 3575 and 6528 nt, respectively, encoding nucleocapsid protein (N), glycoprotein precursor (GPC) and viral RNA-dependent RNA polymerase (L) proteins of 423, 1136 and 2145 amino acids in length, respectively (GenBank accession numbers: KX845678, KX845679, KX845680). Sequence comparison revealed that the three genome segments and the encoded proteins of BRNV showed 54.7–78.3% nucleotide and 44.5–81.7% amino acid sequence identity with other bat-borne hantaviruses while sequence identity with hantaviruses from rodents, shrews and moles ranged between 50.1 and 64.8% at the nucleotide and between 38.9 and 64.1% at amino acid level (Table 1).

The observed amino acid sequence differences clearly exceed one of the current species demarcation criteria of the current International Committee on Taxonomy of Viruses (ICTV) for the genus Hantavirus. Hanta, 7% difference in amino acid sequences of the N and GPC proteins. The current ICTV criteria also include serological differences in virus neutralization, preventing virus classification without a cell culture isolate. However, a new proposal for species demarcation criteria (assigned code of the proposal: 2016.023a;BM; available for download at https://talk.ictvonline.org), submitted by the ICTV Bunyaviridae Study Group, is currently under consideration which allows the classification solely based on genetic data. It is based on a concatenated multiple sequence alignment of complete amino acid sequences of the N and GPC proteins which is used to calculate PED (pairwise evolutionary distances) values using WAG amino acid substitution matrix. A species of the genus Han- tavirus is defined by a PED value greater than 0.1. According to this criterion, BRNV clearly represents a new hantavirus species because the lowest PED value, observed for the most closely related LQVU, is 0.5. The obtained BRNV sequences were subjected to phylogenetic analyses within the Maximum Likelihood framework using MEGA7 (Kumar et al., 2016). Unfortunately, only partial L segment sequences are available for the majority of bat-borne hantaviruses. Therefore, we first based our analysis on a short L segment dataset (352 nt) which contains all currently recognized bat-borne hantaviruses (Fig. 1). BRNV clustered within the clade containing all bat-borne hantaviruses and shared the

### Table 1: Nucleotide and amino acid sequence identities (%) of three genome segments and corresponding encoded nucleocapsid protein (N), glycoprotein precursor (GPC) and RNA-dependent RNA polymerase (RdRp) between Brno virus (BRNV) and other representative bat-, insectivore- and rodent-borne hantaviruses.

| Host       | Virus strain | Country | S segment/N | M segment/GPC | L segment/RdRp |
|------------|--------------|---------|-------------|---------------|----------------|
| **Bats**   |              |         | 1272 nt     | 3411 nt       | 6435 nt        |
| Longquan   | China        | 65.9    | 65          | 663           | 62.5           | 78.3b          | 80.5b          |
| Laibin     | China        | 58.7    | 56.3        | 55.4          | 45.6           | 66             | 66.7           |
| Huangpi    | China        | 65.6b   | 64c         | 61.2          | 54.8e          | 70.1b          | 85.2b          |
| Xuan Son   | Vietnam      | 58.5    | 54.3        | 61.2          | 54.8e          | 70.1b          | 85.2b          |
| Moyausseni | Côte d'Ivoire| –       | –           | –             | –              | 73.4b          | 78.9           |
| Magboi     | Sierra Leone | –       | –           | –             | –              | 74.2b          | 75.7           |
| Makokou    | Gabon        | –       | –           | –             | –              | 66.8a          | 67.6           |
| Quezou     | Philippines  | 59.2    | 55.5        | 54.7          | 44.5           | 65.4           | 66.6           |
| Shrews     |              |         | 424 aa      | 1137 aa       | 2145 aa        |
| Ulgueru    | Tanzania     | 50.9    | 40.9        | 54.8b         | 42.9b          | 64.5           | 62.7           |
| Altai      | Russia       | 56.9b   | 52.6b       | 54.8b         | 48.9b          | 63.3           | 62.3           |
| Cao Bang   | Vietnam      | 57.1    | 51.7        | 51.9          | 40.4           | 63.7           | 62.1           |
| Seewis     | Switzerland  | 57.8    | 49.4        | 54.2b         | 46.9b          | 60.7b          | 60.3           |
| Thottapalyam | India    | 54      | 46.5        | 50.1          | 38.9           | 62.8           | 62.2           |
| Moles      |              |         | 1272 nt     | 3411 nt       | 6435 nt        |
| Nova       | Belgium      | 57.5    | 51.7        | 54.6          | 44.2           | 64.5           | 63.3           |
| Asama      | Japan        | 55.6    | 51          | 52.2b         | 40.3b          | 64.8b          | 64.1b          |
| Rodents    |              |         | 424 aa      | 1137 aa       | 2145 aa        |
| Puamula    | Finland      | 58.3    | 51.9        | 51            | 40.1           | 63.2           | 60.3           |
| Sin Nombre | USA          | 58.8    | 53          | 52.5          | 41.6           | 63.4           | 61.3           |
| Seoul      | Korea        | 55.4    | 48.8        | 51.6          | 39.1           | 62.2           | 60.7           |
| Hantaan    | Korea        | 56.6    | 50.1        | 51            | 40             | 62.2           | 60.6           |
| Dobrava-Belgrade | Greece | 55.7 | 49.8 | 50.4 | 40.1 | 62.5 | 61.1 |
| Tula       | Czech Republic| 57.2    | 52.9        | 51.9          | 40.9           | 63.1           | 61.3           |

- no sequence available.

a Viral sequences used to generate sequence identities: Bat-borne hantaviruses: Longquan virus (JX465415, JX465397, JX465381), Laibin virus (KM102247, KM102248, KM102249), Huangpi virus (JX473273, JX465360), Xuan Son virus (KJ004710, KJ000539, KJ074715), Moyausseni virus (KJ066171), Magboi virus (KJ037851), Makokou virus (KJ161767), Quezou virus (KJ159071, KJ159074, KJ159075), Shrews-borne hantaviruses: Ulgueru virus (JX193695, JX193698, JX193697), Altai virus (KF366024, KF366025, KF366026); Moles-borne hantaviruses: Nova virus (KT004445, KT004446, KT004447), Asama virus (EU929072, EU929075, EU929078); Rodent-borne hantaviruses: Puamula virus (NC_005224, NC_005223, NC_005225), Sin Nombre virus (NC_005215, NC_005217), Seoul virus (NC_005236, NC_005237, NC_005238), Hantaan virus (NC_005218, NC_005219, NC_005222), Dobrava-Belgrade virus (NC_005233, NC_005234, NC_005235), Tula virus (NC_005227, NC_005228, NC_005226).

b Comparison based on shorter sequences.
most recent common ancestor with LQUV found in insectivorous Rhinolophus sp. bats in China. This pattern has been consequently observed in all other phylogenetic analyses including complete amino acid sequences for all three segments (phylogenetic trees based on partial sequences of S and M segments are shown in Supplementary Figs. 1 and 2).

Fig. 1. Maximum-Likelihood phylogenetic tree showing the phylogenetic position of Brno virus (BRNV; marked by black arrow and bold face) constructed on the basis of partial L segment nucleotide sequences (352 nt). Evolutionary analysis was conducted in MEGA7 (15). The evolutionary history was inferred by using the Maximum-Likelihood method based on the General Time Reversible (GTR) model with a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I) which was estimated to be the Best-Fit substitution model according to Bayesian Information Criterion. The scale bars indicate an evolutionary distance in substitutions per position in the sequence. Bootstrap values ≥70%, calculated from 500 replicates, are shown at the tree branches. The insert shows the phylogenetic group containing all bat-borne hantaviruses (marked by bat pictogram) in greater detail. Rodent-borne hantaviruses associated with members of the families Muridae or Cricetidae are indicated by grey-shaded background. The list of the accession numbers used in the analysis is available from the authors upon request. Abbreviations: ALTV, Altai virus; ANDV, Andes virus; ARBY, Ash River virus; ARTV, Artybash virus; ASAV, Asama virus; ASIV, Asikkala virus; ASGV, Azagny virus; BAVY, Bayou virus; BCCV, Black Creek Canal virus; BOGV, Boginia virus; BOWV, Bowé virus; BRNV, Brno virus; CBNV, Cao Bang virus; CDV, Cano Delgado virus; CHOV, Chocol virus; DOBV, Dobrava-Belgrade virus; HOKV, Hokkaido virus; HUPV, Huangpi virus; HTNV, Hantaan virus; JEJV, Jeju virus; JMSV, Jemez Springs virus; KKV, Kilimanjaro virus; KMOV, Kenkeke virus; LAV, Laibin virus; LiEV, Lianghe virus; LNV, Laguna Negra virus; LQGV, Longquan virus; MAKV, Makokou virus; MAPV, Maporal virus; MGBV, Magboi virus; MMOV, Montano virus; MUJV, Muju virus; NVAV, Nova virus; OXEV, Oxbow virus; PHV, Prospect Hill virus; PUUV, Puumala virus; QZNV, Quezon virus; RMOV, Rio Mamore virus; RPKV, Rockport virus; RPLV, Camp Ripley virus; SANGV, Sangassou virus; SEOV, Seoul virus; SERV, Serang virus; SNV, Sin Nombre virus; SWSV, Seewis virus; TANGV, Tanganyika virus; TIGV, Tigray virus; THAV, Thailand virus; TPMV, Thottapalayam virus; TULV, Tula virus; ULEV, Uluguru virus; VAV, Vladivostok virus; XSV, Xuan Son virus. In cases of HTNV, PUUV, SWSV, and XSV, several sequences of the same virus were marked by grey curve to allow unambiguous designation of the taxa.
3. Conclusions

In the present study a novel bat-borne hantavirus has been identified and its complete sequence of all coding genomic regions has been determined. The successful determination of the genome segment sequences of BRNV provide reference data for improving detection methods and determining the genome sequences of further European bat-borne hantaviruses.

Its phylogenetic relatedness to other bat-borne hantaviruses, high genetic distance to other known hantaviruses, and its independent detection in two distinct animals of the same species and in two organs led us to conclude that BRNV is an indigenous bat-borne hantavirus associated with common noctule bat (N. noctula). This study provided a missing molecular proof that bat-borne hantaviruses occur in Europe too, and need to be considered as putative public health threat.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.12.025.

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