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Short communication

Phylogenetic analysis of a newfound bat-borne hantavirus supports a laurasiatherian host association for ancestral mammalian hantaviruses

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ABSTRACT

Until recently, hantaviruses (family Bunyaviridae) were believed to originate from rodent reservoirs. However, genetically distinct hantaviruses were lately found in shrews and moles, as well as in bats from Africa and Asia. Bats (order *Chiroptera*) are considered important reservoir hosts for emerging human pathogens. Here, we report on the identification of a novel hantavirus, provisionally named Makokou virus (MAKV), in Noack's Roundleaf Bat (*Hipposideros ruber*) in Gabon, Central Africa. Phylogenetic analysis of the genomic L-segment showed that MAKV was the most closely related to other bat-borne hantaviruses and shared a most recent common ancestor with the Asian hantaviruses Xuan Son and Laibin. Breakdown of the virus load in a bat animal showed that MAKV resembles rodent-borne hantaviruses in its organ distribution in that it predominantly occurred in the spleen and kidney; this provides a first insight into the infection pattern of bat-borne hantaviruses. Ancestral state reconstruction based on a tree of L gene sequences of all relevant hantavirus lineages was combined with phylogenetic fossil host hypothesis testing, leading to a statistically significant rejection of the mammalian superorder *Euarchontoglires* (including rodents) but not the superorder Laurasiatheria (including shrews, moles, and bats) as potential hosts of ancestral hantaviruses at most basal tree nodes. Our data supports the emerging concept of bats as previously overlooked hantavirus reservoir hosts.

1. Introduction

Hantaviruses are zoonotic pathogens that can cause febrile disease with renal and/or pulmonary failure. Case fatality rates can reach up to 50% (Kruger *et al.*, 2015a). While mainly considered as rodent-borne viruses, new hantaviruses were recently found in African bats. Magboi virus was identified in Slit-faced Bat (*Nycteris hispida*) from Sierra Leone (Weiss *et al.*, 2012) and Mouyassué virus in Banana Bat (*Neoromicia nana*) in Côte d'Ivoire (Sumibcay *et al.*, 2012). Additional hantavirus findings in bats were reported from Vietnam (Arai *et al.*, 2013), China (Guo *et al.*, 2013; Xu *et al.*, 2015), and the Czech Republic (Dufkova L and Ruzek D; personal communication, GenBank accession number KR920360). Spillover infections of bats with rodent-borne Araraquara virus in Brazil were reported as well (de Araujo *et al.*, 2012).

Many bat species live in large and dense social groups that promote virus amplification and maintenance. Their long lifespan, ability of flight, and the ability to occupy diverse habitats including human dwellings, enable efficient pathogen spread. Besides ecological implications, the ability of flight might have physiological consequences that could be linked to bats' special capability to act as reservoir hosts for intracellular pathogens (Brook and Dobson, 2015). Bats are thought to constitute one of the most important mammal reservoir host groups for emerging human pathogens (Calisher *et al.*, 2006; Drexler *et al.*, 2013).

The discovery of hantaviruses in bats could open new insights into their origin and ecology. However, most recent findings were limited to singular infected animals from which relatively small RT-PCR fragments were amplified and sequenced. Here we report on the detection of a new hantavirus in Noack's Roundleaf Bat (*Hipposideros ruber*) from Gabon, including the genetic characterization of its large (L) genome segment by Sanger and next-generation sequencing. Moreover, the distribution of virus RNA within the host organism was determined.

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2. Materials and methods

2.1. Animal study

Within our study, 324 bats (Table 1) were trapped in 2009 in a cave near the city of Makokou, Gabon (Fig. 1). The animals were dissected in the field and the collected blood and organ samples were frozen in liquid nitrogen. The blood samples were used for primary screening with a genus-reactive nested RT-PCR assay, targeting the large (L) genomic segment as described previously (Klempa *et al.*, 2006). In order to determine the tissue tropism of MAKV in the infected bat, a quantitative RT-PCR assay, based on fluorescent hydrolysis probes, was established (Table 2), and performed on a LightCycler instrument.

2.2. Sequencing

To obtain additional genomic sequences, a next-generation-sequencing approach was used. In the reverse transcription step a mixture of random and non-random primers designed to bind to conserved ends was used to over-represent hantaviral sequences. Generated RT-PCR products were purified using the Clean & Concentrator kit (Zymo, Freiburg, Germany). Sequencing libraries were prepared using the Nextera Library Prep Kit (Illumina, San Diego, CA, USA) according to standard protocol. Prepared libraries were sequenced with the Miseq reagent kit V2 (Illumina) using paired end sequencing with 120 bp read length. All resulting sequences were analyzed in CLC Genomics Workbench (Qiagen, Venlo, Netherlands). Pure *de novo* assembly did not lead to identification of any viral contigs. We therefore turned to the "mapping to reference" approach.

2.3. Phylogenetic analysis and ancestral reconstruction

Phylogenetic analysis based on the deduced amino acid sequence of the L segment was performed by using the Maximum Likelihood reconstruction in MEGA v6 (Tamura *et al.*, 2013). Because of restricted sequence length in most published sequences, the only bat-associated viruses that could be included in this analysis were LBV and XSV. To provide a more complete phylogenetic overview, an additional analysis including additional (but shorter) hantavirus sequences, was done by Maximum Likelihood in PhyML (Guindon and Gascuel, 2003).

The PhyML tree was used to perform an ancestral reconstruction of a possible global hantaviral host. We conducted probabilistic hypothesis testing in a Maximum Likelihood framework (Pagel *et al.*, 2004; Drexler *et al.*, 2012; Marklewitz *et al.*, 2015). This approach determines the most likely trait change matrix along the hantavirus phylogeny as well as the loss of likelihood that occurs when restricting traits at given tree nodes. The laurasiatherian or euarchontoglirian host associations were ascribed to tree taxa in the form of binary traits. Using the program Bayestraits (Pagel *et al.*, 2004), a hypothesis-free reconstruction run was performed that clearly identified a laurasiatherian host association at deep tree nodes. This reconstruction was used as a reference dataset to record the median likelihood of host trait change matrices over 1000 bootstrap tree replicates. Fossil host assumptions were then defined at deep tree nodes, restricting the optimization space for the ML algorithm. The resulting loss of Maximum Likelihood for the trait change matrices under restricted conditions was taken as criterion to reject hypotheses, with a greater than 10-fold loss of likelihood being considered significant.

Table 1
Bats collected in Gabon and tested for hantavirus.

Bat species	Common name	No. of positive/collected samples
Hipposideros gigas	Giant leaf-nosed bat	0/137
Hipposideros ruber	Noack's Roundleaf Bat	1/123
Miniopterus inflatus	Greater long-fingered bat	0/64

3. Results

From the 324 investigated bats, a single sample from a Noack's Roundleaf Bat, designated GB303, was found positive. Sanger sequencing followed by NCBI BLAST comparison identified the RT-PCR product as a fragment of a hantavirus genome (data not shown). Primary phylogenetic analysis involving short fragments of the L segment of all so far identified bat-borne hantaviruses, as well as related viruses from shrews and moles revealed that Xuan Son virus (XSV) from Vietnam and Laibin virus (LBV) from China were the most closely related viruses (data not shown). GB303 exhibited the highest nucleotide and amino acid identity (76.2% and 86.2%, respectively) with XSV (Table 3). Although the taxonomically relevant S and M segment sequences are not available, the observed sequence distance of the obtained L segment sequence against other hantavirus species (Maes *et al.*, 2009) suggests that GB303 represents a novel hantavirus, provisionally called Makokou virus (MAKV).

In order to obtain additional genomic sequences, two Illumina Miseq next-generation-sequencing attempts were performed. A total of over 17 million raw reads were generated in two sequencing runs. Pure *de novo* assembly did not lead to the identification of any viral contigs. Attempts to map the reads to any available hantavirus complete S and M segment sequences failed, reducing subsequent phylogenetic analyses to the genomic L segment. All available hantavirus complete L segment sequences were tested but only the sequence of the shrew-borne Thottapalayam virus (NC_010707) allowed some mapping. Altogether 28 reads could be mapped to the reference. They were used for primer design which allowed closing of sequence gaps and additional confirmation by Sanger sequencing. Altogether, this approach led to the extension of the known L segment sequence to 3582 nucleotides, covering the C′-terminal part of the viral RNA-dependent RNA-polymerase ORF (GenBank accession number KT316176). A phylogenetic analysis based on the deduced 1173 amino acid sequence of the L segment is shown in Fig. 2 and confirms the close relationship of MAKV with other bat-borne hantaviruses. The following ancestral reconstruction analyses, summarized in Fig. 3, indicated that 7 out of 9 nodes of the tree as found in Fig. 3A, including the root node, show a high probability to originate from a laurasiatherian ancestor (Fig. 3B). Node F, being a well resolved ancestor node for *Muridae*-borne hantaviruses, is the only node with a high probability for euarchontoglirian origin. Node E, where the *Talpidae*-borne Rockport virus ancestrally clusters with the *Cricetidae*-associated hantaviruses, is the only node within the analysis for which no type of host associations could be clearly rejected. However, this node is not a deep tree node but ancestral to a clade of only rodent-associated viruses, making a rodent association at this node likely.

In the quantitative RT-PCR assay, the virus could be detected in all of the available organs (brain, gut, heart, kidney, liver, spleen). The highest virus loads were observed in the spleen (4.80×10^4 cp/μg RNA), heart (2.97×10^4 cp/μg RNA), and kidney (2.01×10^4 cp/μg RNA) (Fig. 4). The morphological species identification was confirmed by sequencing of the cytochrome-b gene (accession number KT316177). Moreover, all 323 samples being negative within the first nested-PCR-based screening were re-tested using a MAKV-specific quantitative RT-PCR, but remained negative.

4. Discussion and conclusions

The identification of a third, bat-borne hantavirus from Africa together with findings of hantaviruses in bats from Vietnam (Arai *et al.*, 2013), China (Guo *et al.*, 2013; Xu *et al.*, 2015) and Czech Republic (Dufkova L and Ruzek D, personal communication) further supports the emerging concept of bats acting as hantavirus reservoir hosts. The order *Chiroptera* belongs to the most speciose and diversified groups of mammals, representing approximately 20% of classified mammal