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Short Communication

Molecular identification of a tentatively novel hantavirus in Malaysian Bronze Tube-nosed Bat (*Murina aenea*)

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Abstract: In the past ten years several novel hantaviruses were discovered in shrews, moles and bats, suggesting the dispersal of hantaviruses in many animal taxa other than rodents during their evolution. Interestingly, the co-evolutionary analyses of most recent studies have raised the possibility of non-rodents may have served as the primordial mammalian host and harboured the ancestors of rodent-borne hantaviruses as well. The aim of our study was to investigate the presence of hantaviruses in bat lung tissue homogenates originally collected for taxonomic purposes in Malaysia, 2015. Hantavirus specific nested RT-PCR screening of 116 samples targeting the L segment of the virus have revealed the positivity of two lung tissue homogenates originating from *Murina aenea* bat species. Nanopore sequencing of hantavirus positive samples resulted in partial genomic data from S, M and L genome segments. The obtained results indicate the first molecular evidence for hantavirus in *Murina aenae* bat species and also the first discovery of a hantavirus in *Murina* bat species. Sequence analysis of the PCR amplicon and partial genome segments suggests the identified virus may represent a novel species in *Mobatvirus* genus within *Hantaviridae* family. Furthermore, our results provide additional genomic data to help extend our knowledge about the evolution of these viruses.

Keywords: Mulu mobatvirus, MinION, Tb1-Lu, *Mobatvirus*, one health concept

1. Introduction

Hantaviruses (*Hantaviridae*) cause two types of life-threatening human diseases, haemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas [1]. To date, as a consensus, wild rodents were believed as natural hosts of hantaviruses. However, recent studies described several novel hantaviruses in shrews, moles and bats, suggesting the dispersal of hantaviruses in several animal taxa during their evolution [2]. To date, 10 bat-borne hantaviruses were described in different bat species from Hipposideridae, Rhinolophidae, Emballonuridae, Nycteridae, and Vespertilionidae families and only one from a flying fox species Geoffroy's rousette (*Rousettus amplexicaudatus*) [3-4].

Interestingly, phylogenetic analyses of most recent studies have raised the possibility of bats or other animals (shrews and moles) of the Laurasiatheria superorder may have served as the primordial mammalian host and harboured the ancestors of rodent-borne hantaviruses [3,5]. However, complex analyses for the genetic diversity and phylogeography of bat associated hantaviruses are tentative since complete genomic data is available only from Brno virus (BRNV), Dakrong virus (DKGV), Láibīn virus (LAIV), Quezon virus (QZNV) and Xuân Sơn (XSV) viruses. Unfortunately, in case of other bat-associated hantaviruses just partial genomic fragments are available manly from the conservative L segment hardening the implementation of evolutionary analyses [3-5].

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Here we present a presumably novel hantavirus tentatively named Mulu hantavirus (MUV) within *Mobatvirus* genera detected in Bonze tube nosed bat (*Murina aenea*) from Malaysia. Our partial sequences from the S, M and L segments may contribute to the filling of evolutionary gaps and may help to get closer to understand the evolution of hantaviruses.

2. Materials and Methods

2.1 Sample collection and nucleic acid extraction

Lung tissue samples were collected in Gunung Mulu National Park and Gunung Gading National Park, Malaysia in 2015 (Figure 1). Bats were captured by mist nets and harp traps in their foraging habitats. After species identification, specimens which were taxonomically or phylogenetically important were anaesthetized and dissected on site. Tissue samples were stored and transported in Dry Shipper containing liquid nitrogen. Total RNA was extracted from lung tissues using QIAamp MinElute Virus Spin Kit (Qiagen). The field sampling was carried out according to widely approved ethical guidelines of handling mammalian species [6] with research permissions NCCD.907.4.4(JLD.12)-168, 422/2015 and DF.945.201(Jrd.5)-62, and export permit no. 16024, issued by the Controller of National Parks and Nature Reserves, Forest Department, Sarawak, Malaysia.

2.2 PCR screening

All samples were subjected to nested reverse transcription-PCR (RT-PCR) system using previously published, hantavirus-specific universal degenerated nested primer sets [7]. First round PCR reactions were performed by OneStep RT-PCR Kit (Qiagen) with the following cycling conditions: reverse transcription at 50°C for 30 min and 95°C for 15 min, then 40 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 30 s, and elongation at 72°C for 1 min, and final elongation at 72°C for 10 min. Second round PCR reactions were performed using GoTaq G2 Flexi DNA Polymerase Kit (Promega) with an initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 94°C for 1 min, annealing 52°C for 45 s, and elongation at 72°C for 1 min, and final elongation at 72°C for 10 min. PCR products were sequenced bidirectionally using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) on the ABI Prism 310 genetic analyzer platform (Applied Biosystems).

2.3 Metagenomic cDNA preparation and Nanopore sequencing

Prior to Nanopore sequencing, lung tissue homogenates were exposed to enrichment protocol as detailed previously [8], followed by a ribodepletation procedure using The RiboMinus™ Eukaryote System v2 kit (Life Technologies. Complementary DNA (cDNA)). Samples were then subjected for Sequence Independent Single Primer Amplification (SISPA) approach [9]. cDNA amplification was performed by AMV Reverse Transcriptase (Promega) according to the provided manual by the manufacturers using FR26RV-N (5′-GCCGGAGCTCTGCAGATATCNNNNNN-3′) primer. Thereafter, ds cDNA was amplified by DreamTaq DNA Polymerase (Thermo Scientific) according to the supplied protocol using the FR20RV (5′-GCCGGAGCTCTGCAGATATC-3′) primer. Amplified cDNA was purified by NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and quantified using Qubit dsDNA BR Assay kit (Thermo Fisher Scientific). Libraries were prepared using the PCR Barcoding Kit (SQK-PBK004) and protocol by Oxford Nanopore Technologies. R9.4.1 flow cell and MinKNOW v2.0 software were used for sequencing. As the first step of sequence data analysis, adapters were trimmed using Porechop v0.2.4 [10], with default settings. Reads with internal adapters, which were indicating chimera reads, were also splitted (--midle_treshold) with Porechop. For long read alignment, DIAMOND was used against the full NCBI NR database with the following options turned on: -F 15 --range-culling [11]. Mobatvirus related sequences were extracted from the DIAMOND results, and we used these sequences for further analysis.

2.4 In vitro virus propagation

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99
100 *Tadarida brasiliensis* lung tissue cells (Tb1-Lu, ATCC® CCL-88™) were maintained in EMEM
101 (Lonza, Switzerland) supplemented with 10% Fetal Bovine Serum (Biosera, France) and 1% Penicillin-
102 Streptomycin (Lonza, Switzerland) at 37°C with 5% CO_2 until 70% confluency in 24 well plate. 200 µl of
103 supernatant from each hantavirus PCR positive lung tissue homogenate was placed on the cell
104 monolayer and incubated for 1 h at 37°C. Thereafter, cells were supplemented with 1 ml of extra fresh
105 medium and were monitored for cytopathogenic effect for 7 days post-infection. After 7 days, cells were
106 frozen at -80°C and thawed, in order to lyse the cells and 200 µl of the inoculums was used for each
107 additional passages from the previous plates.

108 *2.5 Phylogenetic analyses*

109 Phylogenetic tree reconstruction was implemented using MrBayes v3.2.4 software, with the
110 GTR+G+I substitution model. Analysis settings were as follows: 10 million generations (25%
111 discarded as burn-in), sampled every 1000 generations. The run was stopped after three and a half
112 million generations when the standard deviation of split frequencies was 0.003. Trees were edited
113 using the iTol online tool [12].
114

115 **3. Results and Discussion**

116 *3.1. Virus detection*

117 Hantavirus screening nested RT-PCR was carried out on 116 bat lung tissue samples
118 representing 9 bat families, 15 genera and 33 species (Table S1). Hantavirus RNA was detected in two
119 Bronze Tube-nosed Bat (*Murina aenea*) lung tissue samples from Gunung Mulu National Park,
120 Malaysia (Figure 1.). Unfortunately, all attempts to amplify the complete genome of the three
121 segments of this hantavirus failed, however we applied a combination of multiple primers (Table 1.)
122 as were previously described [13-14]. The attempts to isolate the MUV on Tb1-Lu cell line have failed
123 as well. After the 7th blind passage we could not detect hantavirus RNA in the supernatants of lysed
124 cells by the previously mentioned nested RT-PCR system. The failure of complete genome
125 amplification and isolation of the virus may be due to many factors such as the high level diversity
126 of hantavirus sequences carried by bats, low virus titer in the examined organ or virus RNA
127 degradation as a consequence of improper tissue preservation as it is suggested by previous studies
128 [15, 3]. Despite, *in vitro* isolation of rodent-borne hantaviruses is well established on Vero E6 cells but
129 newly identified hantaviruses carried by bats, insectivore and rodents remain uncultured due to the
130 lack of host-derived cell lines [16]. Nanopore sequencing of MUV positive samples has revealed
131 partial genome sequences of 671, 1326 and 677 nucleotides of the S, M and L segments respectively.

132

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133 **Figure 1.** The distribution area of Bronze Tube-nosed Bat (*Murina aenea*) according to IUCN Red List is marked with red
134 color. Collection sites for the study are marked with blue dots.

135

136 **Table 1.** Oligonucleotide Primers used to amplify the complete S, M and L segments of Mulu hantavirus

Genomic segment	Primer name	Primer sequence (5'-3')	Reference
S,M,L	OSM55F	TAGTAGTAGACTCC	[13]
S	HVSF1	TAGTAGTAGACTCCTTRAARAGC	[14]
	HVSR1906	TAGTAGTAKRCWCCYTRARAAG	
M	HVMF1	TAGTAGTAGACWCCGCAAAAG	
	HVMR3684	TAGTAGTATRCTCCGCARG	
L	HVLF1	TAGTAGTAGACTCCRGA	
	HVLR6561	TAGTAGTAGTAKRCTCCGRGA	

137 *3.2 Sequence and phylogenetic analysis*

138 Pairwise alignment of partial S, M and L sequences of MUV were compared to strains from
139 *Orthohantavirus*, *Mobatvirus*, *Loanvirus* and *Thottimivirus* genera available in GenBank (Figure S1;
140 Table S2). The partial S (671 nucleotides) and partial L (383 nucleotides) sequence of MUV displayed
141 85.7% and 92.1% amino acid sequence similarity respectively against a Laibin virus strain (GenBank:
142 MK393932) from Myanmar. The partial M (1326 nucleotides) sequence of MUV displayed 84.1%
143 amino acid sequence similarity to another Laibin virus strain (GenBank: MK064115) from Myanmar.
144 According to the currently recognized demarcation criterion of International Committee on
145 Taxonomy of Viruses (ICTV) 7% difference in amino acid level of the nucleocapsid protein (NP) and
146 glycoprotein precursor (GPC) is the limit to assign a hantavirus as a tentatively novel species.
147 Although the complete genome could not be amplified, based on the sequence homology data we
148 assume that MUV may represent a new hantavirus species within *Mobatvirus* genera.
149 Maximum likelihood phylogenetic analysis of S, M and L segments of members of
150 *Orthohantavirus*, *Mobatvirus*, *Loanvirus* and *Thottimivirus* (Table S2) genera have revealed MUV
151 segregated into the clade of *Mobatviruses* where MUV is displaying a monophyletic group with Laibin
152 virus. Analyses of all segments suggest that MUV shared a common ancestor with Laibin virus which
153 is its currently known most close relative (Figure 2).

154

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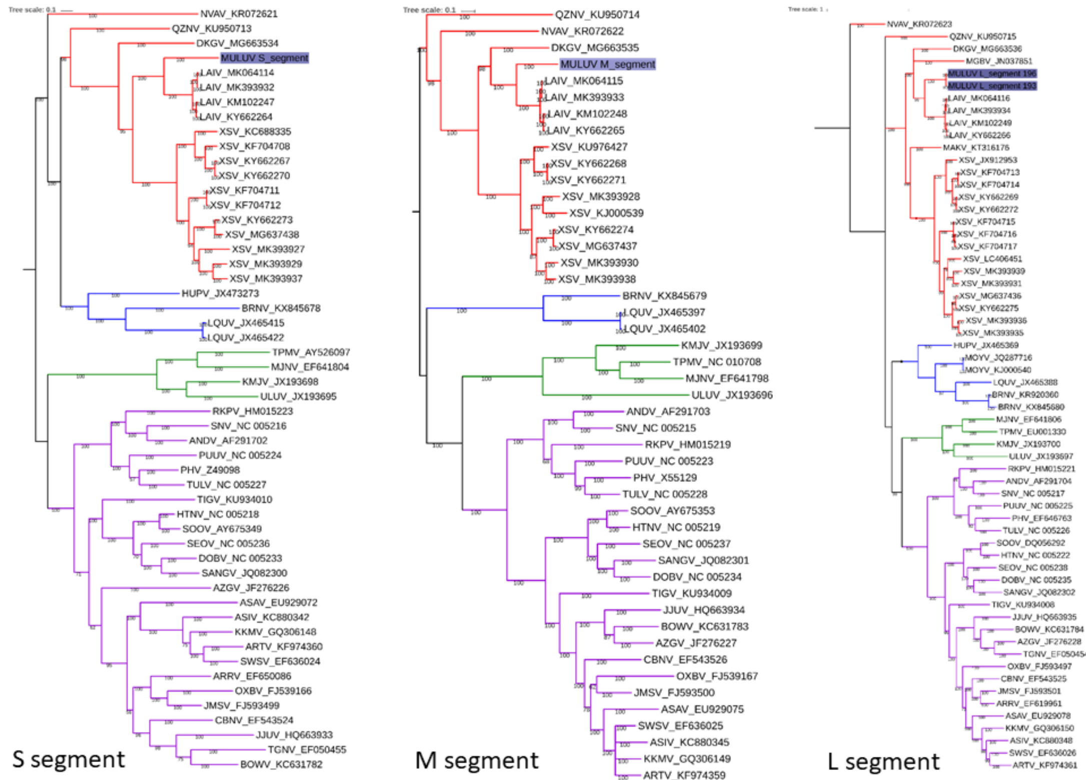

155

156 **Figure 2.** Maximum likelihood analysis of hantavirus S, M and L segments respectively, visualized by iTol online server.
157 Mulu hantavirus reported in this study is highlighted with blue background. Branch color for *Mobatviruses* are highlighted
158 with red, *Loanviruses* are highlighted with blue, *Thottimiviruses* are highlighted with green and *Orthohantaviruses* are
159 highlighted with purple.

160 ## 4. Conclusions

161 Among mammals, bats (Chiroptera) represent the second most diverse order with
162 approximately 1400 species distributed throughout the world except the arctic areas [17]. To date,
163 several evolutionary studies confirmed that bats represent the most ancestral hosts in case of many
164 viruses and they are able to asymptomatically host a range of viruses including highly pathogenic
165 zoonotic agents for humans and animals [18-19]. In the past decades, the growing tendency in the
166 discovery of hantaviruses from bats and the conducted evolutionary analyses on them raised the
167 possibility that bats may be the primordial host of ancient hantaviruses [3].
168 The tentatively novel member of the *Mobatvirus* genus, the MUV was isolated from a rare bat
169 species, *Murina aenea*. This species has a limited distribution restricted to the Malayan Peninsula and
170 Borneo [20]. The bats caught in the Gunung Mulu National Park represent new, unpublished records
171 of the species and highlights the importance of studies on the bats of lowland primary forests
172 following the concept of OneHealth studies. *Murina aenea* may be a foliage-dweller with very limited
173 connection with other bat species. This latter information strengthens the hypothesis that bats served
174 as primordial hosts of ancient hantaviruses since the chance for spillover event connected to this
175 species in the past is very low.

176 **Supplementary Materials:** The following are available online, Table S1: List of bat species examined in this study; Table S2:
177 Hantavirus accession number list used for pair-wise alignment and phylogenetic analysis; Figure S1: Pair-wise alignment of
178 S, M and L segments of viruses in *Hantaviridae* family.

179 **Author Contributions:** Sample collection, taxonomical identification and dissection were made by G.C., T.G., F.A.A.K.,
180 N.F.D.A.T.; laboratory processes were made by B.Z., D.B., G.K., F.F.; preparation of Tb 1Lu cells was made by M.M and H.P;
181 preparation of libraries for Nanopore sequencing was made by G.K. and G.E.T; data analysis of Nanopore sequencing was
182 made by P.U. and R.H.; phylogenetic analysis and Pair-wise alignment were done by G.K. and S.Z.; writing - original draft
183 preparation B.Z., G.K. ; supervision F.J., G.K., G.C., T.G.

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