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Research paper

Candidate new rotavirus species in Schreiber's bats, Serbia

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ABSTRACT

The genus *Rotavirus* comprises eight species designated A to H and one tentative species, *Rotavirus I*. In a virus metagenomic analysis of Schreiber's bats sampled in Serbia in 2014 we obtained sequences likely representing novel rotavirus species. Whole genome sequencing and phylogenetic analysis classified the representative strain into a tentative tenth rotavirus species, we provisionally called *Rotavirus J*. The novel virus shared a maximum of 50% amino acid sequence identity within the VP6 gene to currently known members of the genus. This study extends our understanding of the genetic diversity of rotaviruses in bats.

1. Introduction

Rotaviruses (RVs, family *Reoviridae*, genus *Rotavirus*) are a major cause of acute diarrhea in mammals and birds. At present, eight recognized and one proposed rotavirus species (RVA to RVH and RVI, respectively) are distinguished. Among these, RVA to RVC, RVE, RVH and RVI are known to infect mammals and RVA is the most widespread species in most, if not all, mammalian hosts (Estes and Greenberg, 2013; Matthijnssens et al., 2012; Mihalov-Kovács et al., 2015).

Batborne RVs described so far belong almost exclusively to RVA; sequence analysis of the identified strains uncovered some intriguing details concerning the ecology and evolution of batborne RVAs. For example, a bat strain from Kenya had an unusual VP1 gene and the hypothesis arose that during their evolution mammalian RVs belonging to different RV species may share genes by reassortment (Esona et al., 2010). Furthermore, bats seem to serve as reservoirs of multiple RVA genotypes commonly found in heterologous host species. Consequently, batborne RVAs might pose some veterinary and public health risk (Asano et al., 2016; He et al., 2013; Xia et al., 2014). More recent data indicate that in addition to RVA, RVH may also infect bats (Kim et al., 2016).

Among bats, Schreiber's bat (*Miniopterus schreibersii*) represents one of the most widespread species complex in the world, living in large colonies. Schreiber's bats are distributed in distinct lineages throughout Oceania, Africa, Southern Europe and South-East Asia (Appleton et al., 2004). Colonies of *M. schreibersii* are usually large and dense so that members of the colony can save energy during the hibernation period. These bats may roost together with *Rhinolophus ferrumequinum*, *Rhinolophus euryale*, *Myotis myotis*, *Myotis blythii*, and *Myotis emarginatus*. *M. schreibersii* is able to fly large distances (>500 km) from one roost to another (Hutterer et al., 2005). Overall, these colonial and behavioral characteristics of *M. schreibersii* may notably influence pathogen dissemination that could lead to high prevalence and maintenance of viruses within colonies (Kemenesi et al., 2014).

Our recent pilot study on fecal virome analysis of the Hungarian bat fauna provided new insight into viral diversity, providing evidence of novel astroviruses and bufaviruses in *M. schreibersii* (Kemenesi et al., 2014, 2015). To further explore the ecological role of these common bats as virus reservoirs we involved additional geographical locations

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in our surveys. While we were prepared that new virus diversity may be explored by the method of viral metagenomics, we unexpectedly, identified sequence traces of a novel rotavirus in multiple samples. Sequence and phylogenetic analysis of the complete genome sequence of a selected rotavirus strain provided evidence of a candidate new rotavirus species in these bats.

2. Materials and methods

2.1. Bat guano

Bat guano samples were collected on October 3rd 2014 at cave Pionirska pećina (Beljanica Mt., Serbia; 44° 4′ N, 21°38′ E) during regular bat-ringing activities by experienced chiropterologists (under a license provided by the Ministry of Energetics, Development, and Environmental Protection of the Republic of Serbia, license number: 353-01-2660/2013-08). A mist-net (7 × 2.5 m) was set up at the cave entrance before sunset and remained open until 2 a.m. The trapped bat specimens were removed immediately, identified following Dietz et al. (2009) and held individually in perforated disposable paper bags for maximum of 30 min in order to let them defecate. After collecting fecal samples, bats were aged, sexed, measured, banded and released. A total of 128 *Miniopterus schreibersii* were captured (45 males and 83 females), and fecal samples were collected from ten specimens (3 males and 7 females). Droppings were stored in RNAlater RNA Stabilization Reagent (QIAGEN) and kept on ice until laboratory processing.

2.2. Semiconductor sequencing

Guano samples were homogenized in 500 μL phosphate buffered saline. After 5 min centrifugation in 10,000 ×*g*, 200 μL of the supernatant was used for nucleic acid extraction, performed with GeneJet Viral DNA and RNA Purification Kit (Thermo Scientific Ltd.), following the manufacturers recommendations. Nucleic acid samples were previously denatured at 97 °C for 5 min in the presence of 10 μM random hexamer tailed by a common PCR primer sequence (Djikeng et al., 2008). Reverse transcription was performed with 1 U AMV reverse transcriptase (Promega), 400 μM dNTP mixture, and 1× AMV RT buffer (composition at 1× concentration; 50 mM Tris-HCl [pH 8.3], 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine and 10 mM DTT) at 42 °C for 45 min following a 5 min incubation at room temperature. Then, 5 μL cDNA was added to 45 μL PCR mixture to obtain a final volume of 50 μL and a concentration of 500 μM for the PCR primer (Djikeng et al., 2008), 200 μM for dNTP mixture, 1.5 mM $MgCl_2$, 1× Taq DNA polymerase buffer, and 0.5 U of Taq DNA polymerase (Thermo Scientific). The reaction conditions consisted of an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of amplification (95 °C for 30 s, 48 °C for 30 s, 72 °C for 2 min) and terminated at 72 °C for 8 min. 0.1 μg of cDNA was subjected to enzymatic fragmentation and adaptor ligation following the manufacturers recommendations (available at www.neb.com; NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent™ kit, New England Biolabs). The barcoded adaptors were retrieved from the KAPA Adaptor Kits for Ion Torrent Platforms (Kapa Biosystems). The resulting cDNA libraries were measured on a Qubit® 2.0 equipment using the Qubit® dsDNA BR Assay kit (Invitrogen). The emulsion PCR that produced clonally amplified libraries was carried out according to the manufacturer's protocol using the Ion PGM Template kit on an OneTouch v2 instrument (Life Technologies). Enrichment of the templated beads (on an Ion One Touch ES machine, Life Technologies) and further steps of pre-sequencing set-up were performed according to the 200 bp protocol of the manufacturer. The sequencing protocol recommended for Ion Torrent PGM Sequencing Kit on a 316 chip was strictly followed (Life Technologies).

2.3. Determination of the termini of genomic RNA

To obtain the true sequence of the genome segment ends, a short oligonucleotide (PC3-mod), phosphorylated at the 5′ end and blocked at the 3′ end with dideoxy cytosine, was ligated to the 3′ ends of the genomic RNA in the nucleic acid extract (Lambden et al., 1992; Potgieter et al., 2002). In brief, 5 μL total RNA was combined with 25 μL RNA ligation mixture (consisting of 3.5 μL nuclease free water, 2 μL of 20 μM PC3, 12.5 μL of 34% (w/v) polyethylene glycol 8000, 3 μL of 10 mM ATP, 3 μL 10× T4 RNA Ligase buffer and 10 U T4 RNA Ligase I (New England Biolabs) and then incubated at 17 °C for 16 h. Following the incubation, the RNA was extracted using the QIAquick Gel Extraction Kit (QIAGEN). Binding of RNA to silica-gel column was performed in the presence of 150 μL QG buffer from the extraction kit and 180 μL isopropanol. All subsequent steps were performed according to the manufacturer's instructions.

Five microliter ligated RNA was heat-denatured in the presence of 1 μL of 20 μM primer (PC2-mod, which is complementary to the PC3-mod oligonucleotide ligated to the 3′ end) at 95 °C for 5 min and then placed on ice slurry. The reverse transcription mixture contained 14 μL nuclease free water, 6 μL 5× First Strand Buffer, 1 μL of 10 μM dNTP mixture, 1 μL 0.1 M DTT, 20 U RiboLock RNase Inhibitor (Thermo Scientific) and 300 U SuperScript III Reverse Transcriptase (Invitrogen). This mixture was added to the denatured ligated RNA and incubated at 25 °C for 5 min and then 50 °C for 60 min. The reaction was stopped at 70 °C for 15 min.

Subsequently, 2 μL cDNA was added to the PCR mixture, which consisted of 17 μL nuclease free water, 1 μL of 10 μM dNTP mixture, 2.5 μL 10× DreamTaq Green Buffer (including 20 mM $MgCl_2$), and 2 μL of 20 μM primer pair (i.e. 1 μL PC2 and 1 μL gene-specific primer; see Table 1) and 2.5 U DreamTaq DNA polymerase (Thermo Scientific). Gene-specific primers were designed on the basis of preliminary sequence data obtained by semiconductor sequencing. The thermal profile consisted of the following steps: 95 °C 3 min, 40 cycles of 95 °C 30 s, 42 °C 30 s, 72 °C 2 min, final elongation at 72 °C for 8 min. The PCR products were visualized on 1% agarose gel electrophoresis and bands of the expected sizes were excised and cleaned up with Geneaid Gel/PCR DNA fragments Extraction Kit (Geneaid).

Amplicons were subjected to Sanger sequencing with the PCR primers using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Ethanol precipitated products were run on an ABI PRISM 310 Genetic Analyzer.

2.4. Sanger sequencing of the full-length NSP1 and NSP5 genes

The genome segments encoding NSP1 and NSP5 of RV strains belonging to various RV species may be either mono-, bi- or tricistronic. To validate the results obtained by semiconductor sequencing we performed traditional sequencing. In brief, cDNA production, amplification and Sanger sequencing were carried out with sequence specific primers (Table 1) designed based on the Ion Torrent sequence reads. The experimental protocol was essentially the same as described in the previous section describing the method for determination of genome segment termini.

2.5. RVJ-specific screening RT-PCR assay

Stool samples were homogenized in 500 μL PBS. Following a centrifugation step at 10000 ×*g* for 5 min, the viral RNA was extracted from 200 μL of supernatants using GeneJET Viral DNA and RNA Purification Kit (Thermo Scientific) following the manufacturer's recommendations. Genomic RNA was heat-denaturated at 95 °C for 5 min in the presence of 10 μM gene specific primers. Nested RT-PCR amplification was performed with newly designed primers directed to a 338 nt fragment in the RV VP6 protein region (Table 1). To obtain first round PCR product, 5 μL of the heat-denaturated RNA was