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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 (Minioptrerus 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...
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 Pionilska pečina (Belanica 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 nM random hexamer primer (Djikeng et al., 2008), and 200 μM dATP, dGTP, dCTP, dTTP, and 4 μL of cDNA was subjected to enzymatic fragmentation and adaptor ligation following the manufacturer's instructions. Genomic RNA was heat-denatured at 95 °C 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× DreamTag Green Buffer (including 20 mM MgCl₂), 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 DreamTag 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.

2.3. 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 1 (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.

Five microliter cDNA was then added to the PCR mixture, which consisted of 17 μL nuclease free water, 1 μL of 10 μM dNTP mixture, 2.5 μL 10× DreamTag Green Buffer (including 20 mM MgCl₂), 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 DreamTag 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. RVf-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-denatured 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-denatured RNA was
reverse transcribed and amplified using QIAGEN One-Step RT-PCR Kit (Qiagen) in a 25 μL final reaction volume. The reaction was performed at 50 °C for 30 min, followed by 40 cycles of amplification (each cycle included a denaturation step at 94 °C for 30 s, an annealing step at 50 °C, 45 s, and extension step at 72 °C, 1 min). Second round PCR products were analyzed by electrophoresis in 2% agarose gel in TBE buffer stained with GelGreen and then sequenced in both directions using the protocol referred in previous sections.

2.6. Sequence and phylogenetic analysis

For viral metagenomics, raw sequence reads were trimmed and quality controlled using CLC Genomics Workbench (version 9.0; http://www.clcbio.com). The minimal read length parameter was set to 35. Trimmed reads were taxonomically binned using Diamond v0.8.3 versus NCBI-NR (Buchfink et al., 2015). After classification, the output files were analyzed and visualized by MEGAN6 Ultimate Edition (Huson et al., 2016).

The CLC Genomics Workbench software package was utilized to assemble the genome sequence. After visual inspection of sequence mappings a single consensus sequence was created for all 11 genome segments. Further sequence editing and evaluation were carried out by the GeneDoc (Nicholas et al., 1997) and BioEdit software (Hall, 1999) and then analyzed by similarity search using BLAST (Altschul et

Table 1
Primer sequences used in the study.

| Application | Gene          | Orientation | Sequence (5′-3′) | Amplicon length (bp) |
|-------------|---------------|-------------|------------------|----------------------|
| RNA ligation | Universal     | (Phos)-GGA TCC CGG AAG TTC GG-(ddC)* | –                  |
|             | Universal     | CCG AAT TCC CGG GAT GC*               |                     |
| VP1         | Fw            | CTG CTG AAA CAA TTG TTA ACT GTA CAA  | 270                 |
|             | Fw            | GGA TGG ACT GGT TCA GAA CAA AGC TAT TA | 412                 |
|             | Rev           | TCT CTT GGA TGA TTT GAG ATG GAG | 198                 |
| VP2         | Fw            | GAT GGC GCA GAC TTC GGT ATA C        | 233                 |
|             | Fw            | CTC GAT GCA CAG AGA TTA TTA GTC     | 208                 |
|             | Rev           | GAT TTA ACT AAG CAC AGC AAG TAC TCC | 204                 |
|             | Rev           | CTG TTT CTT CTT TTT AGT AGT CTT     | 280                 |
| VP3         | Fw            | ATG TCG TGG TTT ATA AGA TTA AAT G   | 229                 |
|             | Fw            | AAG AGA TAA TTT CGG CGG GTA CTC     | 302                 |
|             | Rev           | TCA ATC GTA ACG TAG AAT GTC TGC TGC | 186                 |
|             | Rev           | CTT CTA TAA GCA TCA TTT CCC CCC GC   | 222                 |
| VP4         | Fw            | AC CTT TTT TCT TTA CAA ATG GCC A    | 243                 |
|             | Fw            | ACT CAG ATG GGT AAT GGC CAT GCA C   | 270                 |
|             | Rev           | CTA TTA TCT TAT TGG ACA GAG GCT TTA | 330                 |
|             | Rev           | GGA CAC ACC GTC ACT ACC AAA TAA TTC | 376                 |
|             | Rev           | GGT TCC AGC CCA TAA TCC CCA CCA    | 439                 |
| VP6         | Fw            | CAT CTC CGG GCT CCT TTT TTA TG     | 244                 |
|             | Fw            | CTC AAA TGC AAG CCA CAG TAT CA     | 328                 |
|             | Rev           | ACT TGT TCC ATT TTT AGC GGA AGC     | 193                 |
| VP7         | Fw            | CTG TCA ATT CGA TAC TGC ACT TIG TTT ATA A | 133                 |
|             | Fw            | GTG TGA GAA AAG ATT CAT CAC AGC     | 247                 |
|             | Rev           | CCA TAT AAA AAC CAG CAA CAT TAA ATC GC | 260                 |
|             | Rev           | TTT CAT ATG TAA ATC CAC CCA ACA AGG AA | 196                 |
| NSP1        | Fw            | GGG AAA AGA TAA ACA ACT TIG AGT ACT | 110                 |
|             | Rev           | ATC GAA CAA GCA AGC AAA AAA AGC     | 151                 |
|             | Rev           | GGA AAC AAA GCA ACC TTT CTT CTC    | 161                 |
| NSP2        | Fw            | CTG GGG ATA CAT TTT CAT CAA TGT GCA | 174                 |
|             | Fw            | CAA GGA AAG ACA AGC AGG AAA TTA CCA | 231                 |
|             | Rev           | CCT ATT TCC AGC TTT ACC AAC CCC TCG | 237                 |
|             | Rev           | GTC ATT CTC CTA CTT TCG GGT GAT A  | 278                 |
| NSP3        | Fw            | CCA CAC GTT AGA ATG TCT GCA         | 168                 |
|             | Fw            | CAT TCA TCG CTT CAA TAA TCA TCA AGG A | 224                 |
|             | Rev           | CCA ATT ATC AGT TTT TCT CTC AAG TCC | 203                 |
|             | Rev           | GGT CAT TTT CTT TGA AAT TCT TTT CTA | 245                 |
| NSP4        | Fw            | TAA AGA GGA CAT CAT GAT ACT CCA GGA | 120                 |
|             | Fw            | GGG TAA ATA AAA TCT ACA CCA TAC AGG AA | 160                 |
|             | Rev           | ACT CAT ATC TTT AAA ATA TTA TTA TGG CTC CAT A | 178                 |
|             | Rev           | CAA CAC CAT ATG TCC GAG TAT TCC TCC | 208                 |
|             | Rev           | GCC CAA AAC ACT CAC GAA ATG ACA     | 191                 |
|             | Rev           | CCG ACA CCT CCA ACT TGC ATG AGA     | 236                 |
|             | Rev           | ATG CCG CTT CTT CTC GGA GGA        | 162                 |
| NSP5        | Fw            | GCC CAA TTA TAC CTC GCC CTC TTA C   | 200                 |
|             | Fw            | CCA CAC ACC GCA CCA AAA AAA GAC GTA CTA | 1074                |
|             | Rev           | CGC CTC GGT TTT TCC TTG CTC CTT C  | 427                 |
| NSP5        | Fw            | CTC TCT CCA AAA TTA ATC CCT CCA CAA AA | 427                 |
|             | Rev           | CAT GGA GGA GGG TTT TCC CTC GTG TGT TA | 643 (1st round)    |
| Screening   | Fw            | GGA TCT CTA AAT TAT CTC CCA C      | 338 (2nd round)     |
|             | Rev           | CTA CTA TAA ATG AGC AGC CCA        |                    |
|             | Rev           | TTA CAA CAT ATG GCC TCC            |                    |
|             | Rev           | GTT CCA TTC TAG CTT TAT CA         |                    |

*a* These oligonucleotides (referred in the text as PC3-mod and PC2-mod, respectively) were adapted from Potgieter et al. (2002).
cies (incl. RVB, RVG, RVH and RVI). To clarify this ambiguous situation, sequences were the most abundant genomic representatives (98.5%); Rotavirus and gemycircularvirus in sample 6). In one specimen (i.e. sample 6) RV sequences were detected in at least three samples (herpesvirus in sample 1, 3, and 4; astrovirus in sample 1 to 3; coronavirus in sample 4 to 6). Terminal sequences at the 5’ ends showed relatively conserved structure with stable nucleotides at positions 1, 2 and 4 and some variations at positions 3, 5, and 6 (segments 1 and 2, GCCACA; segments 3 and 4, GGCATT; segments 5, 7 and 9, GGAATA; segments 6 and 10, GGCAAA), while at the 3’ ends the variation was less (TAYACCC) (see details in Table 2).

2.7. GenBank accession numbers

The whole genome sequence of strain RVJ/Bat-wt/SRB/BO4351/Ms/2014/G1P1 has been deposited under the following accession numbers: KX756619-KX756629.

3. Results and discussion

To explore the viral diversity six fecal specimens collected from apparently healthy adult M. schreibersii bats were processed for viral metagenomics. In these samples various amounts of sequence reads mapped onto known eukaryotic viral sequences (range, ≪0.1% to 0.9%; Fig. 1). When evaluating the results of viral metagenomics data we need to point out that sample processing did not include virion enrichment step and it is not clear whether each of the relevant sequence reads originate from intact virions. Consequently, the presence of potential endogenous viral sequence elements may have affected the overall landscape of viral diversity. For example retrovirus specific reads, which may represent endogenous viral genomic traits from genomic DNA of the host species, were detected in all samples. Overall the rate of eukaryotic virus specific sequence reads was low, likely because we omitted virus particle enrichment procedures in our sample processing protocol. Nonetheless, various eukaryotic viruses were detected in all six selected fecal samples. Herpesvirus, astrovirus and coronavirus sequences were detected in at least three samples (herpesvirus in sample 1, 3, and 4; astrovirus in sample 1 to 3; coronavirus in sample 4 to 6). Rotavirus and gemycircularvirus sequences were found in two and one samples, respectively (both viruses in sample 1; rotavirus without gemycircularvirus in sample 6). In one specimen (i.e. sample 6) RV sequences were the most abundant genomic representatives (98.5%); however, these sequence reads were distributed among various RV species (incl. RVB, RVG, RVH and RVI). To clarify this ambiguous situation, the library DNA that contained the most abundant RV-specific reads was resequenced at a greater sequencing depth. The resulting >1.3 Million sequence reads were subjected to de novo assembly.

As a result, the consensus genome sequence of strain BO4351/Ms/2014 could be assembled from a total of 36,630 sequence reads at 131 X (segment 3) to 457 X (segment 11) average coverage. Once the consensus rotavirus gene sequences were assembled for all 11 genomic segments, the 5’ and 3’ ends of each segment were validated by an independent method. The resulting genome of BO4351/Ms/2014 was 18,135 bp in length (range, 3533 bp for segment 1 and 620 bp for segment 11). Terminal sequences at the 5’ ends showed relatively conserved structure with stable nucleotides at positions 1, 2 and 4 and some variations at positions 3, 5, and 6 (segments 1 and 2, GCCACA; segments 3 and 4, GGCATT; segments 5, 7 and 9, GGAATA; segments 6 and 10, GGCAAA), while at the 3’ ends the variation was less (TAYACCC) (see details in Table 2).

Each segment had non-translated regions at both 5’ end (length range, 6 to 57 nt) and 3’ end (length range, 20 to 84 nt). Encoded proteins were assigned based on significant hits through the Blast engine and conserved peptide motifs. With this approach we found the equivalents of the major structural (VP1 to VP4, VP6 and VP7) and non-structural (NSP1 to NSP5) proteins of RVs (Tables 2 and 3). The encoded structural and non-structural proteins were assigned to particular RNA segments based on the size of full-length genome segments. Additional putative ORFs were predicted to be encoded on segments coding for VP6 and NSP5; however, these putative proteins shared no conserved protein motifs with those of known from other rotavirus species.

In the phylogenetic analyses cognate sequences of representative RVA to RVH strains were included, except for RVE, for which no sequence information is available. Neighbor-joining and maximum-likelihood trees provided similar topologies, clearly distinguishing clade 1 and clade 2 RV strains. The novel batborne RV consistently clustered with clade 2 RV strains, and in particular, with porcine and human RVH strains. One exception was found when analyzing the NSP4 tree, where the limited bootstrap support at the deepest nodes prevented the separation of the two major RV clades (Fig. 2). Consistent with the phylogenetic analyses, the greatest nucleotide and amino acid sequence identities for the novel batborne RV were seen when compared to reference RVH strains (range, 41 (nt%) and 14 (aa%) for NSP4; 63 (nt%) and 64 (aa%) for VP1) (Table 4).

To place the novel batborne strain, BO4351/Ms/2014, into the latest RV taxonomic framework (Matthijnssens et al., 2012; http://www.ictvonline.org), additional VP6 gene sequences were selected from GenBank to represent a broader genetic diversity of various RV species (Fig. 3). In this analysis, again, BO4351/Ms/2014 was most closely related to the major genetic lineage containing RVH strains (49–50%, aa) and showed lower similarity to other clade 2 RVs (RVB, 39%, RVG, 39%). The
The genetic relationship of BO4351/Ms/2014 to clade 1 RVs was marginal (max. identity with RVC, 17%) (Fig. 4, Table 4). Thus, applying the species demarcation sequence cut-off value, which is 53% identity at the amino acid level, we conclude that the novel batborne RV strain represents a new RV species, tentatively called Rotavirus J (RVJ). The reference strain was therefore designated as RVJ/Bat-wt/SRB/BO4351/Ms/2014/GP1.

To determine whether RVJ infection was common among *M. schreibersii* in the cave under investigation, a nested PCR assay was developed targeting a sequence region that is conserved within the VP6 coding gene of both RVH and RVJ. By adapting the nested PCR assay that amplified a 338 bp long fragment (spanning nucleotide position 137 to 474), another four stool samples were found to be positive for RVJ. All PCR products obtained in the 2nd round PCR were bidirectionally sequenced. The low sequence variation within these short segments (data not shown) suggested the presence of the same virus strain within the colony. Notably, given that RVs have been detected exclusively in birds and mammals, the data presented here suggests that bats may be a true host species of RVJ, although further studies are required to confirm this hypothesis.

### Table 3

Comparison of the genome size and the coding potential of different RV species.

| Genome segment | Rotavirus A, Wa | Rotavirus A, 02V0002G3 | Rotavirus B, Bang373 | Rotavirus C, Bristol | Rotavirus D, 05V0049 | Rotavirus F, 03V0568 | Rotavirus G, 03V0567 | Rotavirus H, J19 | Rotavirus I, KE135/2012 | Rotavirus J, BO4351* |
|----------------|----------------|------------------------|----------------------|---------------------|---------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Size (nt)      | Protein (aa)   | Size (nt)              | Protein (aa)         | Size (nt)           | Protein (aa)        | Size (nt)           | Protein (aa)        | Size (nt)           | Protein (aa)        | Size (nt)           | Protein (aa)        |
| 1              | 3302 VP1       | (1088)                 | 3305 VP1             | (1089)              | 3311 VP1            | (1160)              | 3309 VP1             | (1090)              | 3274 VP1             | (1079)              | 3296 VP1            | (1086)              | 3526 VP1             | (1160)              |
| 2              | 2717 VP2       | (890)                  | 2732 VP2             | (895)               | 2847 VP2            | (934)               | 2736 VP2             | (884)               | 2801 VP2             | (913)               | 2769 VP2            | (904)               | 3014 VP2             | (973)               |
| 3              | 2591 VP3       | (835)                  | 2583 VP3             | (829)               | 2414 VP3            | (763)               | 2283 VP3             | (693)               | 2366 VP4             | (777)               | 2464 VP4            | (738)               | 2364 VP4             | (722)               |
| 4              | 2359 VP4       | (775)                  | 2354 VP4             | (760)               | 2106 VP4            | (744)               | 2166 VP4             | (685)               | 2104 VP3             | (685)               | 2174 VP3            | (694)               | 2352 VP3             | (719)               |
| 5              | 1567 NSP1      | (486)                  | 2122 NSP1            | (577)               | 1276 NSP1           | (577)               | 1356 NSP1            | (395)               | 1872 NSP1           | (574)               | 1791 NSP1          | (547)               | 1295 NSP1           | (574)               |
|                |                |                        |                      |                     |                     |                      |                     |                      |                     |                     |                      |                     |                     |                     |
| 6              | 1356 VP6       | (397)                  | 1348 VP6             | (397)               | 1269 VP6            | (397)               | 1350 NSP3            | (402)               | 1353 NSP3           | (398)               | 1314 VP6           | (396)               | 1267 VP6           | (396)               |
| 7              | 1074 NSP3      | (310)                  | 1089 NSP3            | (304)               | 1179 NSP3           | (347)               | 1270 VP6            | (394)               | 1242 NSP3           | (370)               | 1309 NSP3          | (370)               | 1052 VP6           | (394)               |
| 8              | 1062 VP7       | (326)                  | 1066 VP7             | (329)               | 1007 NSP2           | (301)               | 1063 VP7            | (332)               | 1026 NSP2           | (310)               | 1068 NSP2          | (318)               | 1012 NSP2          | (322)               |
| 9              | 1059 NSP2      | (317)                  | 1042 NSP2            | (315)               | 814 VP7             | (249)               | 107 NSP2           | (312)               | 1025 NSP7           | (316)               | 990 VP7           | (295)               | 825 VP7           | (247)               |
| 10             | 750 NSP4       | (175)                  | 724 NSP4             | (168)               | 751 NSP4           | (219)               | 730 NSP5           | (212)               | 765 NSP5           | (217)               | 706 NSP5          | (218)               | 801 NSP4           | (187)               |
| 11             | 664 NSP5       | (197)                  | 699 NSP5             | (208)               | 631 NSP5           | (170)               | 615 NSP5           | (150)               | 672 NSP5           | (195)               | 678 NSP5          | (169)               | 678 NSP5          | (185)               |
|                |                |                        |                      |                     |                     |                      |                     |                      |                     |                     |                      |                     |                     |                     |
| Total          | 18,501         | 19,064                 | 17,932               | 17,912              | 18,500             | 18,186              | 17,961              | 17,989              | 18,135              | 18,135              | 18,135              | 18,135              | 18,135              | 18,135              |

* Abbreviated name of BO4351/Ms/2014.

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**Table 2**

Assignment and some features of the genome segments of the candidate new bat rotavirus, BO4351/Ms/2014.

| Genome segment | Assignment based on the main gene product | Positions of start and stop codons | Sequences at genome segment termini |
|----------------|------------------------------------------|-----------------------------------|------------------------------------|
|                |                                          | Start | Stop  | 5' end  | 3' end  |
| Segment 1      | VP1                                      | 7     | 3153  | GGCAAA  | TATACC  |
| Segment 2      | VP2                                      | 21    | 2981  | GGCAAA  | TACCC   |
| Segment 3      | VP4                                      | 10    | 2490  | GGCAAA  | TATACC  |
| Segment 4      | VP3                                      | 9     | 2156  | GGCAAA  | TACCC   |
| Segment 5      | NSP1                                     | 50    | 1255  | GGAAT   | TACCC   |
| Segment 6      | VP6                                      | 33    | 1220  | GGCAAA  | TATACC  |
| Segment 7      | NSP3                                     | 49    | 1044  | GGAAT   | TACCC   |
| Segment 8      | NSP2                                     | 59    | 958   | GGAAT   | TACCC   |
| Segment 9      | VP7                                      | 8     | 745   | GGAAT   | TATACC  |
| Segment 10     | NSP4                                     | 27    | 659   | GGAAT   | TACCC   |
| Segment 11     | NSP5                                     | 58    | 555   | GGAAT   | TATACC  |

* Order of genome segments was defined on the basis of their size.
It is important to note that by morphological examination all tested animals were confirmed as adult specimens. Immune competence and pathogenicity need to be clarified for most viruses harbored by bats, although asymptomatic virus shedding seems to be common. Further studies are needed to clarify the pathogenicity, prevalence and effect of the virus on bat colonies. Since bats seem to possess special immune characteristics (Zhang et al., 2013), these features may contribute to an altered response to rotavirus infection and explain the high rate of fecal virus shedding in adult M. schreibersii specimens.

Recent years have witnessed considerable sequence data accumulation in public data bases pointing out the enormous genetic diversity within the Rotavirus genus. Viral metagenomics largely contributed to our understanding of this genetic diversity (Asano et al., 2016; He et al., 2013; Kluge et al., 2016; Li et al., 2011; Marton et al., 2015; Mihalov-Kovács et al., 2015; Theuns et al., 2016; Xia et al., 2014). Until the early 2000s RVA to RVG were considered as the only extant RV species (Estes and Greenberg, 2013; Matthijnssens et al., 2012). Sequence independent amplification followed by cloning and sequencing led to the discovery of a novel human RV species that, together with closely related porcine origin strains, was classified into RVH (Matthijnssens et al., 2012; Wakuda et al., 2011; Yang et al., 2004). A newly described member of the Rotavirus genus, RVI, was identified in the fecal viromes of seals and dogs (Li et al., 2011; Mihalov-Kovács et al., 2015).

In this study we described a novel RV detected in M. schreibersii bats from Serbia in 2014. This novel batborne RV belongs to clade 2 RVs, which also includes RVA, RVG, RVH and RVI (Kindler et al., 2013; Mihalov-Kovács et al., 2015). Of interest, the novel strain was closely related to representative strains of RVH suggesting that these RVs had diverged from a common ancestor. Nonetheless, molecular classification indicated that the Serbian batborne RV strain could be the member of

### Table 4

Percentile nucleotide (nt) and amino acid (aa) sequence based identities between the novel batborne RV strain, BO4351/Ms/2014, and reference RVA-RVD and RVF-RVI strains.

| Encoded protein | RVA | RVB | RVC | RVD | RVF | RVG | RVH | RVI |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|
| **VP1**         | 40 5| 25 5| 58 5| 41 5| 24 5| 41 5| 25 5| 24 5|
| VP2             | 34 5| 54 5| 35 4| 14 3| 36 5| 14 5| 36 5| 14 3|
| VP3             | 38 7| 46 7| 38 5| 19 3| 36 7| 16 4| 36 7| 16 3|
| VP4             | 33 7| 51 7| 35 4| 14 3| 35 7| 12 4| 35 7| 12 3|
| VP6             | 35 7| 50 7| 35 4| 17 3| 34 7| 12 4| 34 7| 12 3|
| VP7             | 38 7| 42 7| 36 6| 16 4| 36 7| 14 4| 36 7| 14 3|
| NSP1            | 32 < | 21 | 30 < | 31 < | 21 < | 31 < | 30 < | 30 < |
| NSP2            | 38 6 | 56 6 | 48 5 | 17 4 | 38 6 | 56 5 | 17 4 | 38 6 |
| NSP3            | 36 4 | 44 4 | 34 3 | 11 2 | 38 4 | 33 2 | 11 2 | 38 4 |
| NSP4            | 34 4 | 36 4 | 32 3 | 12 2 | 34 4 | 32 2 | 12 2 | 34 4 |
| NSP5            | 38 3 | 43 3 | 28 3 | 13 2 | 34 3 | 31 2 | 13 2 | 34 3 |

![Fig. 2. Phylogenetic trees obtained for the genes encoding all major structural proteins (VP1 to VP4, VP6, and VP7) and non-structural proteins (NSP1 to NSP5) with representative strains of RVA to RVI. Alignments were created using the TranslatorX online platform (http://translatorx.co.uk/). Phylogenetic trees were prepared using the maximum likelihood method as implemented in Mega6 (http://www.megasoftware.net/). Bootstrap values are shown at the branch nodes. Calibration bars are proportional to the genetic distance.](image-url)
a novel RV species that we propose here as Rotavirus J. New sequence information of the complete RVJ genome should enable the design of sophisticated nucleic acid based diagnostic assays and the production of recombinant protein for serological assays that will help describe further details about the ecology, epizootiology and evolution of the novel RV. Of particular interest, given that many batborne viruses are capable of causing severe disease in humans it will be important to study whether or not the novel RVJ strains pose any occupational risk for professional chiropterologists or individuals coming into contact with bats and their excreta.
Fig. 4. Similarity plot prepared from amino acid sequences of the VP6 protein. Dashed line indicates the rotavirus species demarcation sequence identity cut-off value determined by Matthijnssens et al. (2012). Color codes are indicated below the plot.

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K. Bányai et al. / Infection, Genetics and Evolution 48 (2017) 19–26

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