Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Infection, Genetics and Evolution 48 (2017) 19–26

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Candidate new rotavirus species in Schreiber's bats, Serbia

Krisztián Bányai [a,*,1], Gábor Kemenesi [b,1], Ivana Budinski [c], Fanni Földes [b], Brigitta Zana [b], Szilvia Marton [a], Renáta Varga-Kugler [a], Miklós Oldal [b], Kornélia Kurucz [b], Ferenc Jakab [b,**]

[a] Lendület Pathogen Discovery Research Group, Institute for Veterinary Medical Research, Centre for Agricultural Research, HAS-Centre for Agricultural Research, Budapest, Hungary
[b] Virological Research Group, Szentágothai Research Centre, University of Pécs, Pécs, Hungary
[c] Department of Genetic Research, Institute for Biological Research "Siniša Stanković", University of Belgrade, Belgrade, Serbia

ARTICLE INFO

Article history:
Received 31 August 2016
Received in revised form 30 November 2016
Accepted 1 December 2016
Available online 6 December 2016

Keywords:
Chiroptera
Viral metagenomics
Semiconductor sequencing
Rotavirus
Astrovirus
Coronavirus
Gemycircularvirus
Retrovirus
Miniopterus schreibersii

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

* Correspondence to: K. Bányai, Lendület Pathogen Discovery Research Group, Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, H-1143 Budapest, Hungária krt. 21, Hungary.
** Correspondence to: F. Jakab, Virological Research Group, Szentágothai Research Centre, University of Pécs, H-7624 Pécs, Ifjúság út 20, Hungary.
E-mail addresses: bkrota@hotmail.com (K. Bányai), jakabf@gamma.ttk.pte.hu (F. Jakab).
[1] These authors contributed equally to this article.

http://dx.doi.org/10.1016/j.meegid.2016.12.002
1567-1348/

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

Table 1
Primer sequences used in the study.

Application	Gene	Orientation	Sequence (5′-3′)	Amplicon length (bp)
RNA ligation 5′ and 3′RACE	Universal		(Phos)-GGA TCC CGG GAA TTC GG-(ddC)[a]	–
	Universal		CCG AAT TCC CGG GAT CC[a]	
	VP1	Fw	CTG CTG AAA CAA TCT TTA AGT GCA A	270
		Fw	GGA TTG ACT GGT TCA GAA CTA AGG TAT TA	412
		Rev	TCT CTT CGA TGA TTT GAG ATG GAG	198
		Rev	TCG TCG CAT TCA TTG GAT GTT TTA A	367
	VP2	Fw	GAT GGC GCA GAC TTC GGT ATA C	233
		Fw	CTC GAT GCA CAG AGA TTA CTC GTC	208
		Rev	GAT TTA ACT AAC CGA AGC AAT TCC TTG TA	204
		Rev	CTG TTT CTG CTT TTG TTG AGT CTC ATT TC	280
	VP3	Fw	ATG TCT CCG TTT AGA TGG ATA CAG C	229
		Fw	AAG AGA TAA TTT CGC CGG GTA CTC	302
		Rev	TCA ATC GTA ACG TAG AAT GTC TGC TGC	186
		Rev	CTT TCA TAA GCA TCA TTT CCC TTC GC	222
	VP4	Fw	ACC CTG TTT TCT TTA CAA ATG CGC A	243
		Fw	AGT CAG ATG GGT AAT GGC CAT GCA C	270
		Rev	CTA TTA TCT TAT TCG AGA GAG GCT TTG TA	330
		Rev	GGA GAG AAC GTC AGT ACC AAA TAA TTT CC	376
		Rev	GGT TTC ACG TCC GAA TAT TCG CCA CCA	439
	VP6	Fw	CAG CTC CGG CGT CGT TTT TAA TG	244
		Fw	CTC AAA TGC AAC CGA CAG TAT CA	328
		Rev	AGT TGT TCC ATT TGT ACG GGA AGC	193
	VP7	Fw	CTG TCA ATT CGA TAC TGC ACT TTG TTT ATA A	133
		Fw	GTG TGA GAA AAG ATT CAT CAC AGC CA	247
		Rev	CCA TAT AAA CAC GAA CAT TTT GAA ATC GC	260
		Rev	TTT CAT ATG TAA ATC CCC TGA ACG AA	196
	NSP1	Fw	GGG AAA AGA TAA ACA ACT TGG AGT GA	110
		Fw	ATC GAA GAA GCA AGC AAA ACA CGA	151
		Rev	GGA AAC AAA GCA ACC ATC TTT CTC TC	161
	NSP2	Fw	CTG GGG ATA GAT TTT TAT CAA TGT GCA	174
		Fw	CAA GGA AAC AGA AAG AGG AAA TTA CCA	231
		Rev	CCT AAT TTC AGC TCT ATC AGC CCC TTG C	237
		Rev	GTC ATT CTC CTA CTG CAT CCT GGA GTA	278
	NSP3	Fw	CCA GAC GTT AGA TTC ATG GCT CCA	168
		Fw	CAG TGA TCG CTT CTA TAG TAA TCA TTG AA	224
		Rev	CCA ATT ATC GAC ATT TTC CTC AAG TCC	203
		Rev	GGT CAT TTC CTT TGG AAT TCT TTT CTT A	245
	NSP4	Fw	TAA AGA GGA CAT CAT GTA ACT CCA GGA	120
		Fw	GGG TAA ATA AAA TCT ACA CCA TAC AGG AA	160
		Rev	AGT CAT ATC TTT AAA GTA TTG CTG CAT CAT A	178
		Rev	CAA CAC CAT ATG TGC GAG TAT TCC TTC	208
	NSP5	Fw	GGC CAA AAC ACT GGA ATC AGC AA	191
		Fw	CGC ACA GCT CCA ACT TCG ATT GGA A	236
		Rev	ATG CCG CGT CGT TTT CTG GAA GGA	162
		Rev	GGC CAA TGA TTT AAC GTC CTC TTC A	200
Sequence verification	NSP1	Fw	CCA CAA ACC GGA CCA AAA GAC GTA CTA	1074
		Rev	CGC CTC GTG TTT TGC TTG CTT CTT C	
	NSP5	Fw	CTC TCT CCA AAA TTA ATC CTT CCA GAA AA	427
		Rev	CAT GGA GGA GCG TTT TCT TCT GTG GTG TA	
Screening PCR	VP6	Fw	GGA TTC TCA AAT TAT CTC CAA C	643 (1st round)
		Rev	GGA AGT TGA ATA AAA CCT GG	
		Fw	CGA TTA CAA CAT TGC TTC	338 (2nd round)
		Rev	GTT CCA TTC TAG CTG TAT CA	

[a] These oligonucleotides (referred in the text as PC3-mod and PC2-mod, respectively) were adapted from Potgieter et al. (2002).

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 an initial denaturation at 95 °C for 15 min, and then by 40 cycles of amplification (each cycle included a denaturation step at 94 °C for 30 s, an annealing step at 42 °C for 30 s, and extension step at 72 °C for 1 min). Nested PCR was carried out with the GoTaq DNA Polymerase (Promega). In brief, 3 μL of the first round PCR products were amplified with inner primers for 35 cycles under the following conditions: initial denaturation at 95 °C for 2 min, followed by 40 cycles of amplification (denaturation, 94 °C, 30 s; annealing, 50 °C, 45 s; extension, 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

al., 1990). Multiple alignments were prepared using the TranslatorX online program (Abascal et al., 2010) and manually adjusted in the GeneDoc software (Nicholas et al., 1997), whereas phylogenetic analysis by the maximum-likelihood and the neighbor-joining methods were performed by using the MEGA6 software (Tamura et al., 2013).

For the maximum-likelihood tree the best fit nucleotide substitution models for each gene were selected based on the Bayesian information criterion as implemented in the MEGA software (in particular, TN93 + G was used for NSP3; HKY + G was used for NSP1, NSP4 and NSP5; GTR + G was used for NSP2, VP1, VP2, VP4, VP6, and VP7; GTR + G + I was used for VP3). For the neighbor-joining tree we used the *p*-distance algorithm.

The coding potential was predicted by using the ORF Finder online platform (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

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, GGCACA; segments 3 and 4, GGCATT; segments 5, 7 and 9, GGAAAT; 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

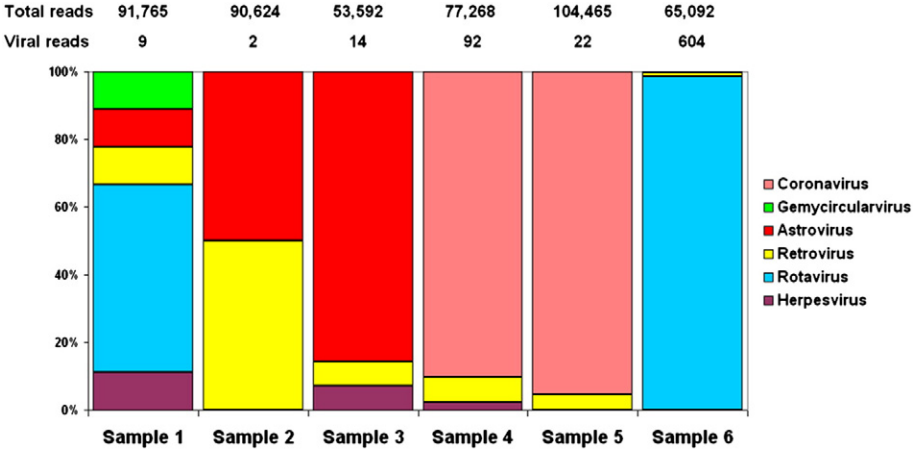

Fig. 1. Distribution of viral sequence reads in six bat fecal specimens.

Table 2
Assignment and some features of the genome segments of the candidate new bat rotavirus, BO4351/Ms/2014.

Genome segment[a]	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	3513	GGCACA	TATACCC
Segment 2	VP2	21	2981	GGCACA	TACACCC
Segment 3	VP4	10	2490	GGCATT	TATACCC
Segment 4	VP3	9	2156	GGCATT	TACACCC
Segment 5	NSP1	50	1255	GGAAAT	TACACCC
Segment 6	VP6	33	1220	GGCAAA	TATACCC
	ORF-X	118	789		
Segment 7	NSP3	49	1044	GGAAAT	TACACCC
Segment 8	NSP2	59	958	GGAAAA	TACACCC
Segment 9	VP7	8	745	GGAAAT	TATACCC
Segment 10	NSP4	27	659	GGCAAA	TATACCC
Segment 11	NSP5	58	555	GGAATT	TATACCC
	ORF-Y	212	433		

[a] Order of genome segments was defined on the basis of their size.

genetic relationship of BO4351/Ms/2014 to clade 1 RVs was marginal (max. identity with RVC, 17%) (Fig. 4, Table 4). Thus, applying the official 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/G1P1.

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.

	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[a]	
Genome segment	Size (nt)	Protein (aa)	Size (nt)	Protein (aa)	Size (nt)	Protein (aa)	Size (nt)	Protein (aa)	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)	3511	VP1 (1160)	3309	VP1 (1090)	3274	VP1 (1079)	3296	VP1 (1086)	3526	VP1 (1160)	3538	VP1 (1167)	3518	VP1 (1162)	3533	VP1 (1168)
2	2717	VP2 (890)	2732	VP2 (895)	2847	VP2 (934)	2736	VP2 (884)	2801	VP2 (913)	2769	VP2 (904)	3014	VP2 (991)	2969	VP2 (973)	3002	VP2 (982)	3010	VP2 (986)
3	2591	VP3 (835)	2583	VP3 (829)	2341	VP3 (763)	2283	VP3 (693)	2366	VP4 (777)	2246	VP4 (738)	2364	VP4 (772)	2512	VP4 (823)	2371	VP4 (777)	2512	VP4 (826)
4	2359	VP4 (775)	2354	VP4 (770)	2306	VP4 (750)	2166	VP4 (744)	2104	VP3 (685)	2174	VP3 (694)	2352	VP3 (768)	2204	VP3 (719)	2161	VP3 (701)	2200	VP3 (715)
5	1567	NSP1 (486)	2122	NSP1 (577)	1276	NSP1–1 (107) NSP1–2 (321) NSP1–3 (65)	1353	VP6 (395)	1872	NSP1 (574)	1791	NSP1 (547)	1295	NSP1–1 (106) NSP1–2 (324)	1307	NSP1 (395)	1485	NSP1–1 (79) NSP1–2 (390)	1322	NSP1 (401)
6	1356	VP6 (397)	1348	VP6 (397)	1269	VP6 (391)	1350	NSP3 (402)	1353	VP6 (398)	1314	VP6 (396)	1267	VP6 (391)	1287	VP6 (396)	1278	VP6 (395)	1277	VP6 (395) ORF-X (223)
7	1074	NSP3 (310)	1089	NSP3 (304)	1179	NSP3 (347)	1270	VP6 (394)	1242	NSP3 (370)	1309	NSP3 (370)	1052	NSP3 (300)	1004	NSP3 (297)	1018	NSP2 (301)	1108	NSP3 (331)
8	1062	VP7 (326)	1066	VP7 (329)	1007	NSP2 (301)	1063	VP7 (332)	1026	NSP2 (310)	1068	NSP2 (318)	1012	NSP2 (282)	932	NSP2 (262)	954	NSP3 (273)	1017	NSP2 (299)
9	1059	NSP2 (317)	1042	NSP2 (315)	814	VP7 (249)	1037	NSP2 (312)	1025	VP7 (316)	990	VP7 (295)	825	VP7 (247)	820	VP7 (258)	858	VP7 (268)	793	VP7 (245)
10	750	NSP4 (175)	724	NSP4 (168)	751	NSP4 (219)	730	NSP5 (212)	765	NSP4 (127) ORF2 (93)	706	NSP5 (218)	801	NSP4 (187)	739	NSP4 (213)	751	NSP4 (219)	743	NSP4 (210)
11	664	NSP5 (197) NSP6 (92)	699	NSP5 (208)	631	NSP5 (170)	615	NSP4 (150)	672	NSP5 (195)	678	NSP4 (169)	678	NSP5 (181)	649	NSP5 (176)	593	NSP5 (157)	620	NSP5 (165) ORF-Y (73)
Total	18,501		19,064		17,932		17,912		18,500		18,341		18,186		17,961		17,989		18,135	

[a] Abbreviated name of BO4351/Ms/2014.

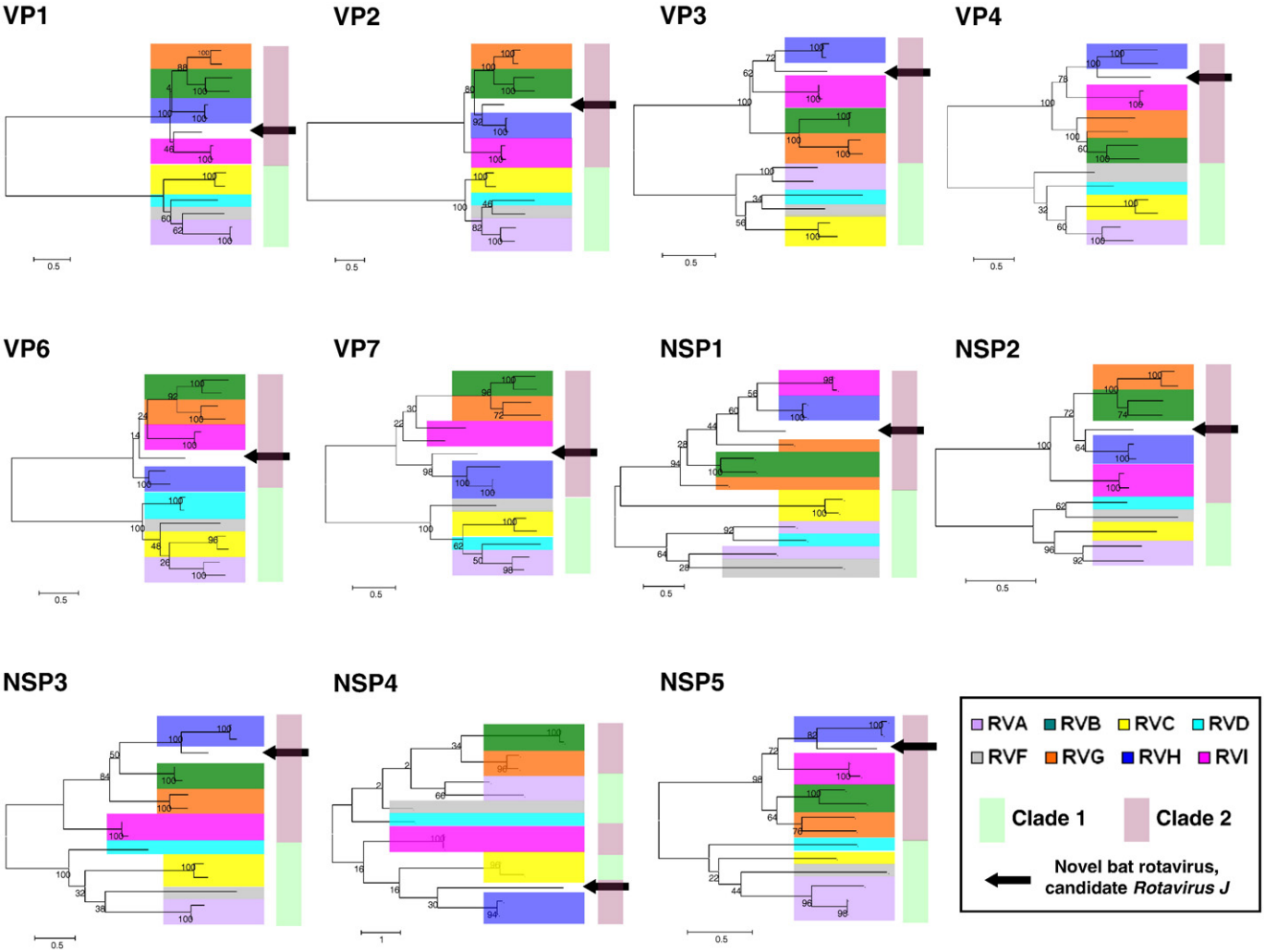

Fig. 2. Phylogenetic trees obtained for the genes encoding all major structural proteins (VP1 to VP4, VP6, and VP7) and non-structural proteins (NSP1 to NSP5) with representative strains of RVA to RVI. Alignments were created using the TranslatorX online platform (http://translatorx.co.uk/). Phylogenetic 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.

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 seems 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 RVB, 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	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
VP1	40	25	58	57	41	24	41	25	42	24	60	59	63	64	61	59
VP2	34	14	54	46	35	14	34	12	36	14	56	47	62	61	54	45
VP3	38	17	46	32	38	19	36	16	38	18	46	31	56	49	51	36
VP4	33	11	41	25	35	14	35	12	35	12	42	25	48	34	44	26
VP6	35	15	50	39	34	17	34	13	32	11	49	39	55	49	52	46
VP7	38	16	42	22	36	16	36	14	37	16	43	25	52	37	49	29
NSP1	32	< 10	39	21	30	< 10	31	< 10	31	< 10	39	18	47	34	44	26
NSP2	38	16	56	48	35	17	38	17	38	17	55	47	63	59	56	46
NSP3	36	15	44	27	34	11	38	18	33	11	40	26	56	49	40	22
NSP4	34	10	36	15	32	12	34	12	33	12	36	10	41	14	35	13
NSP5	38	13	43	28	38	13	34	11	32	11	47	25	50	39	46	31

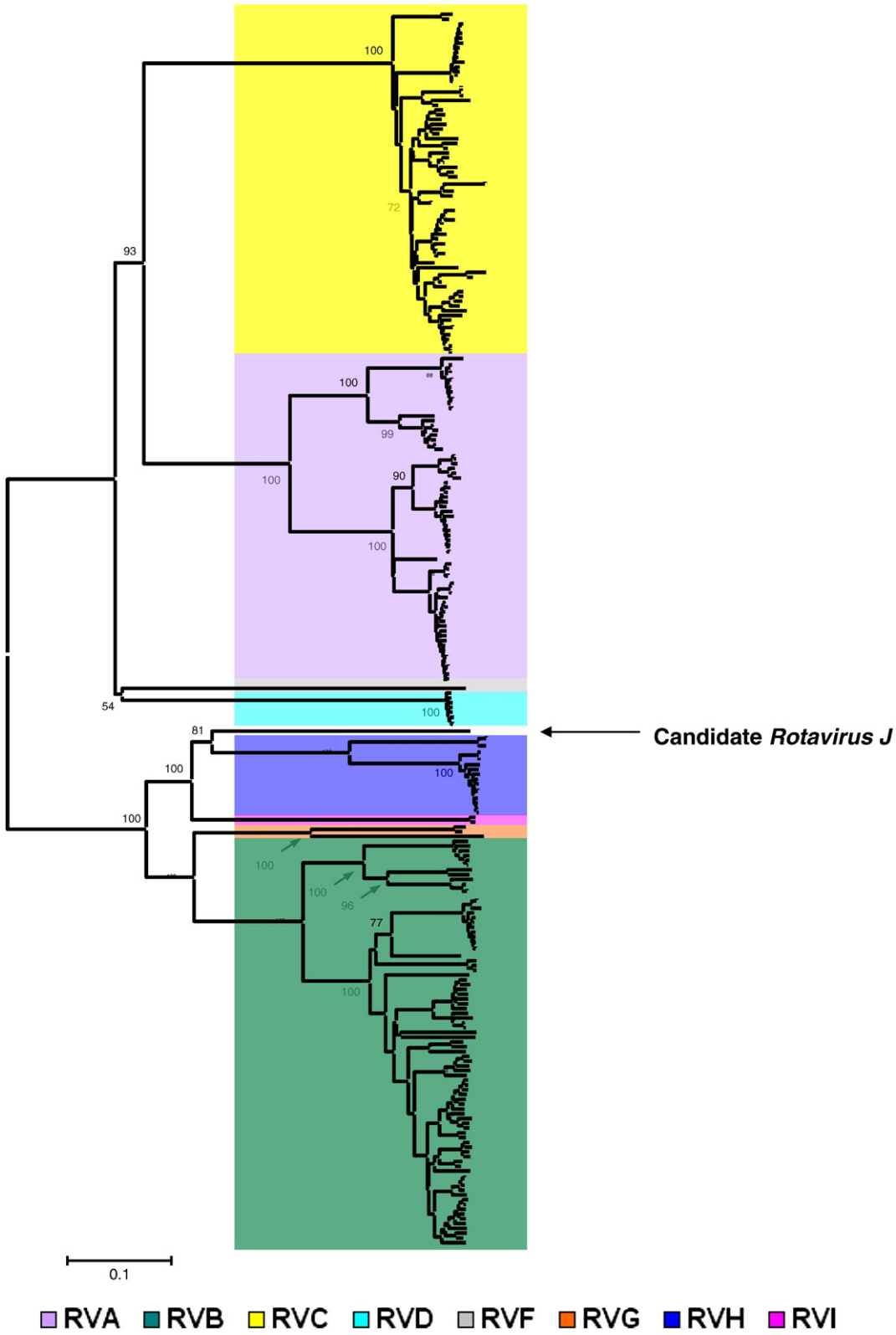

Fig. 3. Phylogenetic analysis of the VP6 gene. A total of 258 representative amino acid sequences were selected to provide a more comprehensive phylogenetic analysis for the VP6 gene. Color codes are indicated below the tree. Bootstrap values at the deeper nodes are shown. Calibration bar is proportional to the genetic distance.

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.

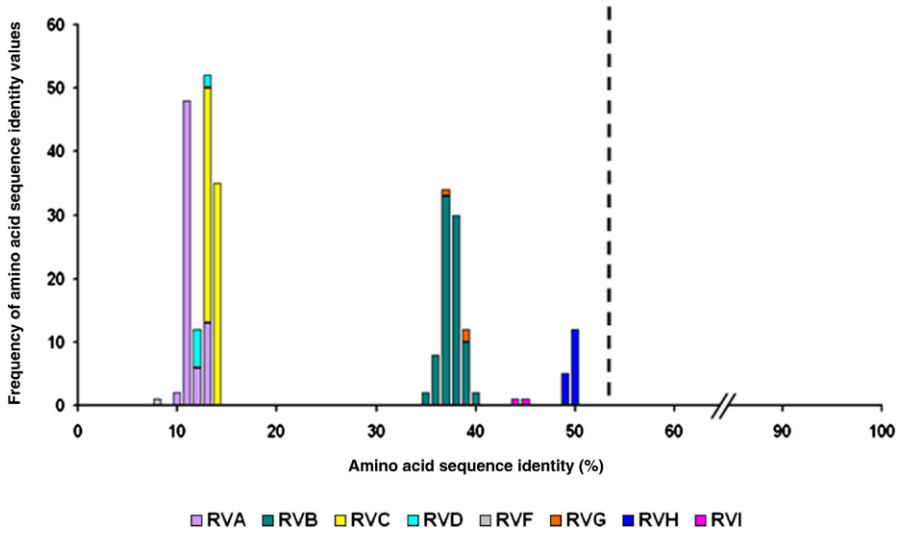

Fig. 4. Similarity plot prepared from amino acid sequences of the VP6 protein. Dashed line indicates the rotavirus species demarcation sequence identity cut-off value determined by Matthijnssens et al. (2012). Color codes are indicated below the plot.

Acknowledgements

Financial support was obtained from the Momentum (Lendület) Program (awarded by the Hungarian Academy of Sciences), from the Ministry of Education, Science and Technological Development of Serbia (Grant No. 173003) and from TÁMOP (4.2.4.A/2-11-1-2012-0001). S.M. was a recipient of the János Bolyai fellowship (awarded by the Hungarian Academy of Sciences), K.K. was supported by the Szentágothai Talent Program (awarded by the Szentágothai Research Centre, University of Pécs). Research activity of G.K. and F.J. was supported by the ÚNKP-16-3-III and ÚNKP-16-4-III – New Excellence Program of the Ministry of Human Capacities. The present scientific contribution is dedicated to the 650th anniversary of the foundation of the University of Pécs, Hungary.

References

Abascal, F., Zardoya, R., Telford, M.J., 2010. TranslatorX: multiple alignment of nucleotide sequences guided by amino acid translations. Nucleic Acids Res. 38, W7–13.
Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Appleton, B.R., McKenzie, J.A., Christidis, L., 2004. Molecularsystematics and biogeography of the bent-wing bat complex *Miniopterus schreibersii* (Kuhl, 1817) (Chiroptera: Vespertilionidae). Mol. Phylogenet. Evol. 31, 431–439.
Asano, K.M., Gregori, F., Hora, A.S., Scheffer, K.C., Fahl, W.O., Iamamoto, K., Mori, E., Silva, F.D., Taniwaki, S.A., Brandão, P.E., 2016. Group A rotavirus in Brazilian bats: description of novel T15 and H15 genotypes. Arch. Virol. 161, 3225–3230.
Buchfink, B., Xie, C., Huson, D.H., 2015. Fast and sensitive protein alignment using DIAMOND. Nat. Methods 12, 59–60.
Dietz, C., von Helversen, O., Nill, D., 2009. Bats of Britain, Europe and Northwest Africa. A and C Black Publishers Ltd., London, UK.
Djikeng, A., Halpin, R., Kuzmickas, R., Depasse, J., Feldblyum, J., Sengamalay, N., Afonso, C., Zhang, X., Anderson, N.G., Ghedin, E., Spiro, D.J., 2008. Viral genome sequencing by random priming methods. BMC Genomics 9, 5.
Esona, M.D., Mijatovic-Rustempasic, S., Conrardy, C., Tong, S., Kuzmin, I.V., Agwanda, B., Breiman, R.F., Banyai, K., Niezgoda, M., Rupprecht, C.E., Gentsch, J.R., Bowen, M.D., 2010. Reassortant group A rotavirus from straw-colored fruit bat (*Eidolon helvum*). Emerg. Infect. Dis. 16, 1844–1852.
Estes, M.K., Greenberg, H.B., 2013. Rotaviruses. In: Knipe, D.M., Howley, P.M., et al. (Eds.), Fields virology, sixth ed. Wolters Kluwer - Lippincott Williams & Wilkins, Philadelphia, pp. 1347–1401.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.
He, B., Yang, F., Yang, W., Zhang, Y., Feng, Y., Zhou, J., Xie, J., Feng, Y., Bao, X., Guo, H., Li, Y., Xia, L., Li, N., Matthijnssens, J., Zhang, H., Tu, C., 2013. Characterization of a novel G3P[3] rotavirus isolated from a lesser horseshoe bat: a distant relative of feline/canine rotaviruses. J. Virol. 87, 12357–12366.
Huson, D.H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., Mitra, S., Ruscheweyh, H.J., Tappu, R., 2016. MEGAN community edition - interactive exploration and analysis of large-scale microbiome sequencing data. PLoS Comput. Biol. 12, e1004957.
Hutterer, R., Ivanova, T., Meyer-Cords, C., Rodrigues, L., 2005. Bat Migrations in Europe—A Review of Banding Data and Literature. Bundesamt für Naturschutz, Bonn, p. 180.
Kemenesi, G., Dallos, B., Görföl, T., Boldogh, S., Estók, P., Kurucz, K., Kutas, A., Földes, F., Oldal, M., Németh, V., Martella, V., Bányai, K., Jakab, F., 2014. Molecular survey of RNA viruses in Hungarian bats: discovering novel astroviruses, coronaviruses, and caliciviruses. Vector Borne Zoonotic Dis. 14, 846–855.
Kemenesi, G., Dallos, B., Görföl, T., Estók, P., Boldogh, S., Kurucz, K., Oldal, M., Marton, S., Bányai, K., Jakab, F., 2015. Genetic diversity and recombination within bufaviruses: detection of a novel strain in Hungarian bats. Infect. Genet. Evol. 33, 288–292.
Kim, H.K., Yoon, S.W., Kim, D.J., Koo, B.S., Noh, J.Y., Kim, J.H., Choi, Y.G., Na, W., Chang, K.T., Song, D., Jeong, D.G., 2016. Detection of severe acute respiratory syndrome-like, Middle East respiratory syndrome-like bat coronaviruses and group H rotavirus in faeces of Korean bats. Transbound. Emerg. Dis. 63, 365–372.
Kindler, E., Trojnar, E., Heckel, G., Otto, P.H., Johne, R., 2013. Analysis of rotavirus species diversity and evolution including the newly determined full-length genome sequences of rotavirus F and G. Infect. Genet. Evol. 14, 58–67.
Kluge, M., Campos, F.S., Tavares, M., de Amorim, D.B., Valdez, F.P., Giongo, A., Roehe, P.M., Franco, A.C., 2016. Metagenomic survey of viral diversity obtained from feces of Subantarctic and South American fur seals. PLoS One 11, e0151921.
Lambden, P.R., Cooke, S.J., Caul, E.O., Clarke, I.N., 1992. Cloning of noncultivatable human rotavirus by single primer amplification. J. Virol. 66, 1817–1822.
Li, L., Shan, T., Wang, C., Côté, C., Kolman, J., Onions, D., Gulland, F.M., Delwart, E., 2011. The fecal viral flora of California sea lions. J. Virol. 85, 9909–9917.
Marton, S., Mihalov-Kovács, E., Dóró, R., Csata, T., Fehér, E., Oldal, M., Jakab, F., Matthijnssens, J., Martella, V., Bányai, K., 2015. Canine rotavirus C strain detected in Hungary shows marked genotype diversity. J. Gen. Virol. 96, 3059–3071.
Matthijnssens, J., Otto, P.H., Ciarlet, M., Desselberger, U., Van Ranst, M., Johne, R., 2012. VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. Arch. Virol. 157, 1177–1182.
Mihalov-Kovács, E., Gellért, Á., Marton, S., Farkas, S.L., Fehér, E., Oldal, M., Jakab, F., Martella, V., Bányai, K., 2015. Candidate new rotavirus species in sheltered dogs, Hungary. Emerg. Infect. Dis. 21, 660–663.
Nicholas, K.B., Nicholas Jr., H.B., Deerfield, D.W.I.I., 1997. GeneDoc: analysis and visualization of genetic variation. Embnet News 4, 14.
Potgieter, A.C., Steele, A.D., van Dijk, A.A., 2002. Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA genes. J. Gen. Virol. 83 (Pt 9), 2215–2223.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Theuns, S., Conceição-Neto, N., Zeller, M., Heylen, E., Roukaerts, I.D., Desmarets, L.M., Van Ranst, M., Nauwynck, H.J., Matthijnssens, J., 2016. Characterization of a genetically heterogeneous porcine rotavirus C, and other viruses present in the fecal virome of a non-diarrheic Belgian piglet. Infect. Genet. Evol. 43, 135–145.
Wakuda, M., Ide, T., Sasaki, J., Komoto, S., Ishii, J., Sanekata, T., Taniguchi, K., 2011. Porcine rotavirus closely related to novel group of human rotaviruses. Emerg. Infect. Dis. 17, 1491–1493.
Xia, L., Fan, Q., He, B., Xu, L., Zhang, F., Hu, T., Wang, Y., Li, N., Qiu, W., Zheng, Y., Matthijnssens, J., Tu, C., 2014. The complete genome sequence of a G3P[10] Chinese bat rotavirus suggests multiple bat rotavirus inter-host species transmission events. Infect. Genet. Evol. 28, 1–4.
Yang, H., Makeyev, E.V., Kang, Z., Ji, S., Bamford, D.H., van Dijk, A.A., 2004. Cloning and sequence analysis of dsRNA segments 5, 6 and 7 of a novel non-group A, B, C adult rotavirus that caused an outbreak of gastroenteritis in China. Virus Res. 106, 15–26.
Zhang, G., Cowled, C., Shi, Z., Huang, Z., Bishop-Lilly, K.A., Fang, X., Wynne, J.W., Xiong, Z., Baker, M.L., Zhao, W., Tachedjian, M., Zhu, Y., Zhou, P., Jiang, X., Ng, J., Yang, L., Wu, L., Xiao, J., Feng, Y., Chen, Y., Sun, X., Zhang, Y., Marsh, G.A., Crameri, G., Broder, C.C., Frey, K.G., Wang, L.F., Wang, J., 2013. Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. Science 339, 456–460.