

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.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active. 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 <sup>a,\*,1</sup>, Gábor Kemenesi <sup>b,1</sup>, Ivana Budinski <sup>c</sup>, Fanni Földes <sup>b</sup>, Brigitta Zana <sup>b</sup>, Szilvia Marton <sup>a</sup>, Renáta Varga-Kugler <sup>a</sup>, Miklós Oldal <sup>b</sup>, Kornélia Kurucz <sup>b</sup>, Ferenc Jakab <sup>b,\*\*</sup>

<sup>a</sup> Lendület Pathogen Discovery Research Group, Institute for Veterinary Medical Research, Centre for Agricultural Research, HAS-Centre for Agricultural Research, Budapest, Hungary

<sup>b</sup> Virological Research Group, Szentágothai Research Centre, University of Pécs, Pécs, Hungary

<sup>c</sup> 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.

© 2016 Elsevier B.V. All rights reserved.

# 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

<sup>\*</sup> 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.

<sup>\*\*</sup> 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).

<sup>&</sup>lt;sup>1</sup> These authors contributed equally to this article.

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  $(7 \times 2.5 \text{ m})$  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  $\times$  g, 200  $\mu$ L 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  $\mu$ M dNTP mixture, and 1  $\times$  AMV RT buffer (composition at 1× concentration; 50 mM Tris-HCl [pH 8.3], 50 mM KCl, 10 mM MgCl<sub>2</sub>, 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<sub>2</sub>,  $1 \times$  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<sup>™</sup> 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  $\mu$ L of 20  $\mu$ M 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  $\mu$ L nuclease free water, 6  $\mu$ L 5× First Strand Buffer, 1  $\mu$ L of 10  $\mu$ M dNTP mixture, 1  $\mu$ L 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  $\mu$ L cDNA was added to the PCR mixture, which consisted of 17  $\mu$ L nuclease free water, 1  $\mu$ L of 10  $\mu$ M dNTP mixture, 2.5  $\mu$ L 10 × DreamTaq Green Buffer (including 20 mM MgCl<sub>2</sub>), and 2  $\mu$ L of 20  $\mu$ M primer pair (i.e. 1  $\mu$ L PC2 and 1  $\mu$ L 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  $\mu$ L PBS. Following a centrifugation step at 10000  $\times$ g for 5 min, the viral RNA was extracted from 200  $\mu$ L 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  $\mu$ M 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  $\mu$ L of the heat-denaturated RNA was

K. Dullyul et ul. / Injection, Genetics und Evolution 46 (2017) 13-2	K. Bányai et al.	/ Infection,	Genetics	and Evolut	ion 48	(2017)	) 19–26
--	------------------	--------------	----------	------------	--------	--------	---------

Table 1			
Drimor coquences	used	:	the

Primer sequences used in the study.

RNA ligation 5' and 3'RACE       Universal       CR0 AAT CC CGC GA AT TC GG-(ddC) <sup>4</sup> -         VP1       Fw       CIG CG AAA CAA CGT TT AA GG GA A       270         VP1       Fw       CIG CG AAA CAA CGT TT AA GG GA A       120         Rev       TC TC CG CAA CT AG GA GA GG AG GA       188         Rev       TC TC CG CAA CT AG GA GA GG CG AG TT GA
UniversalCCG AAT TCC CGG GAT CC <sup>1</sup> VP1FwCGG CTG AA AGAA CTA TTA AGT GGA A270FwCGG TTG AGT GGT TG AGA CTA AGG TAT TA412RevTCT CTT CG AA CTA TTA GGA TG GAG198VP2FwCTG CG CA TTG ATT GAG ATG GAG CTA TA367VP2FwCTG CG CA CA CTA GA TTG CTG CA283RevGAT TGA AGT GA CGA AGA CTA ATT CG TC286VP3FwGAT GCC GA AGA CTA ACT CGA TTG CA TTG CA TTG CA290VP3FwATG TCT CGC TTT AGA TGG ATA CAG CG229VP4CTG TTT CG TA AGT GA AGA TG CT CGC CG TTG CG302RevCTG TT CT AAA TT GGA CAA CG CA CC220VP3FwAGG GAT ATT TG CGC TG CG CG CG CG CG CG220VP4FwAGC GAT ATT TG CG CT GT CGC302RevCTG TT CT TA CAA TG GC CA CC221VP4FwACC CG TT TT CT TA CAA TG GC CA233RevCTG TT TT TT TG CG CA GA CG CG CT CGC A243RevCTG TT TG TT CG AAA CG ACA CC330RevGGT TT CA TT CG AAA GA GA CG CA CG CA330RevGGT TT CA TT GC AAC CGA CAC CC333RevGT TG CAA TT CG CA CC CA CAA330RevGT TG CAA TC CG AAC CC CAC330RevGT TG CAA TC CG ACC TT TG AT TG AACC328RevGT TG CAA TG CG CAAC CAC CC CAC330RevGT GT GAA GCA AAC AACC AAC331RevGT GT GAA CGA AAC AACA CAA CC CAAC341NSP1FwGG GG AAA CAAA CAA CAA CAC CAAC331
VP1FwCIGCIGCIGACAATCITIGZ70FWCGACGA GAACAAACIGAIGAIGRevTCICIGCGAACGAAGAIGAIGRevCIGCGACCGAGCGAGAIGA
FwGGA TIG ACT GGT TCA GAA CTA AGG TAT TA412RevTCT CTT CGA TGA TIT GAA ATG GAG198RevTCC TIT CGA TGA TIT GAA ATG GAG233VP2FwGAT GGA CGA GAA CTT CGT C208RevGAT TGA CGA GAA GAA TTA CT CTT GT A204RevGAT TTA AT CAAC GGA AGA TTA CT GTC208RevGAT TTA AT CAAC GGA AGA TTA CT GTC208RevGAT TTA AT CAAC GGA AGA TTA CT CTG TA204RevGT TTA GA TTG CT TG TG TC CT CT GT AT204RevGT TTA GA TTG CT CGA TG CAGA CAAC C229PWAGA GAGA TAA TTT GC GT CT CT CT CT CT302RevGT TTA CT AAT GC TAG CG CG CAAT CAC C220PWAGA GAGA TAA TTT CC CTT CG CGC302RevCTT CTA TA GCA TC GT CT CT CT CC302RevCT TA CT AAT CG CAAT GCA TAA CC CC TT GT CT GC CG303RevGGA TCA CT GC GAAT CGC CAAT CGC CA239PWGGA TG CA GT GC GAAT TAA TTT GC CAC CG303RevGGA TCA CGT CGA CG ATT GG CC CAAT CGC CG303PWGGA TCA CGT CGA CG CAT TG CGA CGA330PWGGA TG CA CGA CGC CGA CGC CG TT GT TAT CAA333PWGTG TG CGA AAA GA TT CG CC CGA CGC CGA328PWGTG TG CGA AAA CGA TT CG CC CGA CGC CGA328PWGGA CAAA CGT AA CGC CAA CGC CAA330PWGGA CAAA CGT AAA CAAC TT CG CGA CGC CGA330PWGGA CAAA CGA CAA CGA CAA CGC CAA330PWGGA CGA CAAA CGA CAA CGC CAA CGC CAAC331PWGTG
RevTCT CTT CGA TGA TTT GAG ATG GAG198RevTCG CG CGA TGA TTG GAA GT TTA A367VP2FwGAT GGC GCA GAG ATT GC GAT GCA TTA C233FwCT CGA TGC GA GAG ATA TA CT CG TC208RevGAT TTA A CT AAC CGA GAG ATA TTC CT TG TA280PWAGG TGC CGA GGA GAT TTA CT ACC TG GA GGC ATA CAC299PWAGG AGA TAA TTT GGC GG TA GAT CG CA CAC229RevCTG TT CG TA GA TGC ATA CAC CAC222RevTCA ATC GTA AG TG CATA CAC C222PWAGG AGA TAA TTT CGC CGG GTA CTC302RevCTG ATC TAA AGT GAT CTC TCC TGC186RevCTG TA CT AA GG CAT AAT CGC CAC243RevCTG ATA CT AAT GG CAC GAC243PWAGT CTA ATT CTG ATA GG CAC GAC300RevCTA TTA CT ATA TGC ATA GGC CAC GAC330RevCTA TTA CT ATA TGC ATA GGC CAC GAC330RevCTA TAT CTA TAT CAC AAA TAA TTT CC376PWCTG CAC ATA TT CG CAC CAC CAC GAC339PWFWCTG CAC ATAT TGC CAC CAC CAC GACPWCTG CAC ATAT CCC CC CAC CAC CAC GAC330RevCTG TATA CT CAC ACC CAC CAC CAC GAC GAC GAC GAC GAC
RevTCC TCC CAT TCA TTC ATT CAT TTA A367VP2FwCAT CGC CAC CAC CAT TCA CT CAT CAC233FwCTC CAT CGC CAC CAC CAC TTG CT ATA C208RevCAT TTA ACT CAC CGA ACC ACA TTCC TTG TA204RevCTG TTT CTT TTT TTT CTA TTA ACC280VP3FwATC CTT TTG TT TTG TCA TTC CC280VP3FwATC CGT ACG AAC CAAC TTCC TTG TA204RevCTG TTT ACT ATC GAT ACGA ACG CAC229VP4FwATC ATT CGT TA CGA TA CGA CAC222VP4FwATC ACG TAA GCA AT CGT CTC CGC222VP4FwACC CTG TT TTCT TTA AAGC CAC CC270FwACC CTG TT TTCT TTA ATA ATG CGC ACC270RevCTA TAT TTT TTA TTA ATG CGC CAC CC270RevCGA TAA CGC AAC GCAT CCA CC270RevCGA TTA CCT ACC AAAT TAC CACA CC300RevCGA TTA CCT AAC CGC AC CCC CC CC CC CT TT TA300RevCGA TAA TCC ACC CCA AAAT TAC CCAC241PWCTC AAAT CCAC CCC AAA TAA CCC320PWCTC AAAT CCAC CCC CAC ACA TTA CC320PWCTC AAAT CCAC CCC AAAT TTT CAC TAT CC330PWCTC AAAT CCAC CCC AAA CAAC ACC CCC330PWCTC AAAT CCAC CCC CAG AAC CAC CCC320PWCTC AAAT CCAC CCC CAG AAC CAC CCC320PWCTC AAAT CCAC CCC CAAA ACA ACC ACC CCC330PWCTC AAAT CCAC CCC CAG AAC CAC CCC320PWCTC CCAAT ATT CCC CCC CCC CCC320PWCTC CCAA CCCC
VP2FwGAT GCG CAA GAC TITA GCT AAC CGA AGA TITA CT233RevCTG CAT GCA AGA GA TITA CTC GCT CC208RevCTG TITA ACT AAC CGA AGA TITA CCT GC ATT TC204VP3FwATG TCT CG CTT TTG TA CA GG CCA ATT CC TTG TA204VP3FwATG TCT CG CTT TTG TA CATG GA TA CAG C229FwACA GAG TAA TTT GCA CGG GTA CCG302RevCTT TCA TAA GCA TAA TTT CCC TGC TGC TGC302PWACA CAG TAA TTT CCC TGC CGG CTA CCA232VP4FwACC CGT AAT GCG CAG CA CGA CAG243PWACT CAG ATG GT TA TTC CCA TCG CAG243PWACG CGA AAC GTC ATT GCG CAG CAG CAG CAG303PWGGT TTC ACG TAAT GCG CAG CAG CAG CAG CAG303PWGGT TTC ACG TC GAG ACG ACG TTC TG TAT A303PWGGT TTC ACG TC CGA ATT CGC ACG CAG439PWCGG CTT CC CGG CCT CGT TTT TAT CG CGA CGA CAG328PWCGG CTA ATT CGC CGA CCG CAG CAG CAG CAG328PWCTG CAG AAA CGA TT CGC ACG CAG328PWCGG AAA AGA TT CGC CG CAG CAG CAG328PWCGG AAA AGA TT CGC CGA CGA CAG CT331PWCGG AAA AGA TT CGC CGA CAG CAG328PWCGG AAA AGA TT CAG CGA AGC CAG328PWCGG AAA AGA TT CGC CGA CGA CAG328PWCGG AAA AAA CG CGA ACG CAG CGA TT CG328PWCGG AAA AAA AG CGA ACA CAG CGG AGC328PWCGG AAA AAA ACA CGA ACA CAG CGG TG CGA328PWCGG AAA AAAA TT CGC CGA CGA CGA CGA CGA C
FwCTC GAT GCA CAG AGA TTA CTC GTC208RevGAT TTA ACT AAC CGA AGC AAT TCC TTG TTA204RevCTG TTT G G GT TTG AGT GCA AGT CAA TTC CTG GT280VP3FwATG CTC G CTT TG AGT GCA GG CAG C299RevCTG TAT CTC GG TA ATT TCC CCG GG CTA CTC302RevTCA ATG GTA ACG TAA GG TA CG TG CG CG222RevCTT TCA TAA GG ATA TGC TGC TGC280VP4FwACC CTG TTT TCT TTA CAA ATG CGC A243RevCTT TTC TA CAA ATG CG CAA TGC CAA CG AG243PWACC CTG TTT TCT TTA CAA ATG GCC AGT CGA CAA243RevGG AGG AAC GTC AGT ATG CC CAA TA TGC CAA CGA ATG GC CAT TG CA300RevGG AGG AAC GTC AGT ATG CCAA CAA TAA TTT CC CTG AG376PWCG AGG AAC GTC AGT AGG CAA TTG CC AG CAA CGA ATG CG CAA CGA390RevGG TTC ACG TC GG CG CGT TTT TTA TAA330RevCTG CAAA TGC AAC CGA CAG CAA CGA AGC393PWCTG CAAA TGC AAC CGA CAC TT CAA328RevCTG TG AA ATG AAC CG CAA CGC393PWCTG CAAA AGA ATT CAA CGC CAA CT TG TTA TAA133PWCTG CAAA AGA ATT CAA CG CAA CGC394PWCTG CAAA AGA ATT CAA CG CAA CG CAA136PWCTG CAAA AGA ACA CGAAA CAA CGC394PWCTG CAAA AGA ATT CAA CGC CAA136PWCTG CAAA AGA ATT CAA CGC CAA136PWCTG CAAA AGA ATT CAA CGC CAA136PWCGA CGA AAA CGA AACA CGC AAA136PWCGA CGA AAA CGA AAC AACA CGAAA13
RevGAT TTA ACT CAC CAC ACC AT TCC TTG TA204RevCTG TTT GT TTG ACT TACC ATG CTTC TTG CATT TC280VP3FwATG TC TC CG TTT AGA TGG ATA CAG C229RevAG GAG TAA TTT CGC GG GTA CTC302RevCTT TG TA AG CA TGC TGC TGC CG221VP4FwACC TGC TAG CTG CG CG GTA CTC222VP4FwACC TG CTT TCT TTA CAA AGG CCA TCC221RevCTT TG TA AG GG TAAT GGC CAT CGC A243RevCTA TTA TCT TA TTG CG AAT GC CAT CCA C270RevGG TT CA CG CC GG CAT CCA C270RevGG TT TC ACG CAT AGC CAT CAC AC330RevGG TT TC ACG CC CG CAT CCA C439VP6FwCCA CT CCG CAT TT TCG CCA CCA439VP6FwCTG CAA TT CG CAC CCA CAC AGC330VP7FwCTG TG CA AT CG CAA CCA CA CAC CAC AGC330VP7FwCTG TAA CT CG AAT CG CAA CGA CAC CAC AGC330VP7FwCTG TG CAA AGC AAC CAC TAT CAA328VP7FwCTG TG GA AAC CAC CAC CAC TAT AA333RevCTG TG AAA CAC CAC CAC CAC AGC331VP7FwCG CAAT AAAC CAA CAC ACT TTG CAC CAC CAC260NSP1FwCG CAAT AAAC CAAC CAC ACT TTT CAA ATT CC260NSP2FwCTG CG AAA CAAA CAC ACT TTG CAC CAC331RevCTA CGA ACC AAA CAAA CAC ACT TTG CAC CAC231RevCTA CT AAA CAC CAAA CAAA CAC CAC331RevCTA CGA ACC AAA CAAA CAC ACT CTG CAC CAC331
RevCTG TTT CTG CTT TTG TTG ACT CTC ATT TC280VP3FwATG TCT CCG TTT GA TCG CTG CTT ATT CC229FwAAG AGA TAA TTT CGC CGG GTA CTC302RevCTG TA AC GT CA ATT CT TG CTG CC222VP4FwACC CTG TTT CCT TTC CTG CC222VP4FwACC CTG TTT CT TTA GCA TCG CA CC243FwACC CTG TTT CT TTA CAA ATG CGC AT CGA C270FwACC CTG TTT CAT TTC CG CA CGA CG CG CT TTG TA330RevCTA TTA TTC TT TT CT CAA CG ACA CGT TTG TA330FwCGT TTC ACT CGA CT CGA TAC CAAA TAA TTT CC36RevGGT TTC CGT CG CT ACT ACC AAA TAA TTT CC36PWCGC TCC CGA CT CGA TAC CAAA TAA TTT CC36RevGTTC CCG CGT CGT TTT TAA TG34PWCTC CGA CT CGA CAC CAA CAC CAA38PWCTC CGA CT CGA CAC CT TT TAA TG32PWCTG TC AAT TCG AAA CGC CAA CAC CAC32PWCTG TC AAT TC CAAT CG CAC CAC CAA33PWCTG TC AAT TC CAAT CG CAC CAC CAC32PWCTG TC AAT TC CAAT CG CAC CAC CAC32PWCTG TC AAT TC CAAT CC CAC CAC CAC33PWCTG CG AAA CAAT AAA CAC CAAA CAC TT CG AT CG260PWCCG CAT AT AA CAC ACAT CG CAC CAC31PWCTG CGG AAA CAAT TAA ACAC CGAA CAC CAAA31PWCTG CGG AAA CAAT AAA CAC ACAT ACGA CAC31PWCTG CGG AAAC ACAT CTT CAT CCC CGG CT CG33PWCTG CGG AAAC ACAT CTT CAT CCC CAAT CTT CAT31PW<
VP3FwAG G TCT CCG TTT AGA TGG ATA CAG C229FwAGA GAA TAA TTT CGC CGG GTA CC302RevTCA ATC GTA ACG TG AGA AT GCT CC TCC186RevCTT TCA TAA CGA TAG TTT CCC TTC C222VP4FwAGT CGA ATG GCT AAT GCC A243FwAGT CGA ATG GCT AAT GCC AT CGA C270RevCTA TTA TCT TAT TCC AGA GCG CT GCA C270RevGGT ACT CGA ACG GCT ACT AAT TTT CC376RevGGT TTC ACG TCC GAA TAT TTC CC CA CAA339VP6FwCCC AAT CCAA CGA CGT ACT CAA ATTA TTT CC376RevGTT TC ACG TCC GAA TAT TCG CCA CCA39VP6FwCTC CAA TCC ACG CAG GTAT CA328RevGT GT CC ATT CGA TCG GCA AGC CCA193VP7FwCTG TCA ATT CGA TCAC CAT TTG TTT AT A133RevCTG TAA ACG CAA CACT TTG GTT AT A133PWCTG CAA ACG CAA CACT TTG GCA AAC244FwCTG TCA ATT CGA TCAC CAC CC CA247FwCTG TCA ATT CGA TCAT CCAC CC CA247FwCTG CAA AAC ACA ACA CCAC CAC TTT G TTT AT A133FwCGA AAA ACA ACA CAC ACA CCC CA260FWCTG CAA ACA CACA ACA CTT GG AGT GA110FWGG AAA CAAA ACA ACA TT GC ACG CAC231FWCTG CAAA CACA ACA CAC CGA CAC CAA231FWCTG CAAA CACA ACA CACA CCC CCT GC237FWCTG CAAA CACA ACA CACA CCC CCT GC237FWCTG CAAA CACA CACA CACA CCC CCT GC231FWCCA ACG CTT ACG CT C
FwAGA GAA TAA TTT CGC CGG GTA CTC302RevTCA ATC GTA ACG TAG AAT GTC TGC TGC186RevCTT TCA TAA GCA TCA TTT CCT TTC GC222VP4FwACC CTG TTT TCT TTA CAA ATG CGC A243FwACC CTG TTT TCT TTA CAA ATG CGC A243FwCTA TTA TCT TAT TCC GAG CAG CT TTG TA330RevCTA TTA TCT TAT TCC CAG CAG CTTTG TA330RevGGA GAG AAC GTC AGT ACC AAA TAA TTT CC376RevCGC GC TTC CGA TAT TGC CCA CCA439VP6FwCGC GC CT CTC TTT TAA TTG244FwCTC CAA TCC GA CTC CTC TTT TTAA TA328RevCTC TAAT CG ATA CG CAA CG GA ACC328PWCTC CAA CTG CAA TAA TTG GT TATA A330PWCTC CAA ATT GCA TAC CCA CAA247FwCTG TGA GAA AAG ATT CAA TCG CCA CAA244FwCTG TGA GAA AAG ATT CAA TCG CCA CAA328PWCTG CGA GAA AGA CTT CAA TCG CAA328PWCTG CGA GAA AGA CTT CAA CAC CAA CAC CAA247FwCTG TAA TT CAA TCG CCA CAA247PWCTG CGA AAA CAA CAA CAA CAA CAA CAA CAA CA
RevTCA ATC GTA ACG TAG AGT GTC TGC TGC186RevCTT TCA TAG CGA TGC GTT TGC CC222VP4FwACC CTG TTT TCT TTA CAA ATG CGC A243FwAGT CGA GTG GGT AAT GGC CAT GCA C240RevGGA GAG ATG GT CGT CAT GCA CA C270RevGGA GAG ACG CTC AGT ACC AAA TAA TTT CC376RevGGT TTA TCT TAT TCG CGA CAC AAA TAA TTT CC376RevGGT TC CGG CCT CGT AAT GCA CAC CCA CAA439VP6FwCTC CAAA TCC AAA CCGA CAC CAAA TAA TTT CC328PWCTC CAAA TCC AAA CCGA CAC CAAA TAA TT CA328PWCTC CAAA TCC AAA CCGA CAC CAC ACA TAA CAA328PWCTG TCA AAT CCAAA CCGA CAC CAA TAA AT CAA328PWCTG TCA AAT CCAAA CCGA CAC CAA TAA CAA328PWCTG TCA AAA CAA CT GAT CAC ACC CAA TAA AT CAA328PWCTG TCA AAA CAA CT GAT TCA CAC GCA328PWCTG TCA AAA CAA CT GAT TAC CAA TGC AAA328PWCTG TCA AAA CAA CT GAT TAT CAA ACC CAC ACC AAA340PWCTG TCA AAA CAA CT GAA TT TAT AA330PWCTG TCA AAA CAA CAA CAA CAC CAAA CC CAC CAAA341PWCTG TAA TA CAA CAA CAA ACA CAC CAAA TT CAA CT CAC CAC
RevCTT TCA TAA GCA TCA TTT CCC TTC GC222VP4FwACC CGT GTTT TTA CAA ATG CGC A243FwAGT CAG ATG CGT CAT GCA C270RevCTA TTA TCT TAT TCG AGA GAG GCT TTG TA330RevGGA GAA CAC CTA ACT ACA ATA TTT CC376RevGGT TC ACG TCC GAA TAT TCG CCA CA439VP6FwCTC CAA TCC GAA CCG AAC CTA ATG244FwCTC AAA TCC AAC CGA CAG TTT CAG CTA CA328RevGGT TGT TCC ATT TCG ACG CAG CT TT TTA A TG244VP6FwCTC AAA TCC AAC CGA CAG TAT CA328RevAGT TGT TCC ATT TGT ACG GGA AGC193VP7FwCTG TCA ATT CCA TAC TGC ACT TTG TTA TA A133RevCCA TAT CCA ACA CGA ACA CGA CG CA247RevCCA TAA ACAC GAA CAT TTT GAA ATC CCC TGA CG CA246NSP1FwCGG AAA GAA AGA TAA ACA CAT TTT GAA ATC CCC TGA ACG CA110FwGGG AAA GAA GA AGA AGA ACG AA CGA AGA TG TG CA151FwCTG GGG ATA ACAA ACA CGA ACG CAA151FwCTG GGG ATA ACGA AGA GA GGA GAA TTA CCA231FwCTA GGA AAC GAA AGA AGA AGA AGA ATA ACA CGA231FwCCA AGT ATT CAG CT CT TG CAA237RevCTC ATT TC AGT CT CT AGT GG ATA CGA CAA234FwCCA GGG CT AGA CT CT TG CAA CT CT GGA CT CT237RevCTC ATT TC CAG TTC CT CAA CT CT GGA CT CT237RevCCA GGG CT AGA TTT CCAA TTT CCAA TTT CCAA238FwCCA GGG CT TT CT TA GAA TCA CT CAA CT CAA TTG CAA238<
VP4FwACC CTG TTT TCT TTA CAA ATG CGC A243FwAGT CAG ATG GGT AAT GCC CAT CGA C270FwCAT TTA TCT TAT TCG AGA GAG GCT TTG TA330RevCGA GAG AAC GTC ACT ACC AAA TAA TTT CC376RevGGT TTC ACG TCC GAA TAT TCG CCA CCA439VP6FwCTC AAA TGC CAC CGA CAG TAT CA238RevCAG TTG TCC ATT TGC ACG GGA AGC193VP7FwCTC AAT CG AAC CGA CAG TAT CA238RevAGT TGT TCC ATT CGA TAC TGC AGT CAC CAG247RevCTG TGA ATA CGA CAC CAG CAG CAG247RevCCA TAT AAA CAG CAA CAT TTG AAT CG CAG260RevTTT CAT ATG TAA ATC CCC TGA AAC CGA196NSP1FwCGG AAA GAA TAA ACA ACT TGG AGT GA110RevCGA AAA GCA ACA ACA CAA TTT CGA CGA151RevCGG AAA CGA ACG AAC CAA TTT TCT CT CT161NSP2FwCCT GA GG AAA GGA AGC AAT TAT CGA CGA237RevCTC AAT TCT CG CAT CT GG GAT CAC CAG CT TG237RevCTC ATT TAT CAC GG CAT CCT TG CA237RevCTC ATT TT CAT CG CAT CT CG GA CTA CAT TG GA278RevCCA ATT ATC CAC ATT CAT CG CCC CAG237RevCCA ATT ATC AGG CCC CT GG CAT236RevCCA ATT ATC GAC ATT TG CT CAG GAT CCA237RevCCA ATT ATC CAC ATT TG CT CG GA GTA278RevCCA ATT ATC CAC ATT TG CT CCAG GAT CCA237RevCCA ATT ATC CAC ATT TG CT CCAG GAT236RevCCA ATT ATC GAC ATT TG CT CCAG GATA
FwAGT CAG ATG GGT AAT GGC CAT GCA C270RevCTA TTA TCT TAT TCC AGA GAG GCT TTG TA330RevGGG AGA GAC GTC AGT ACC AAA TAT TTCC376RevGGT TTC ACG TCC GAA TAAT TTCC GCA CCA439VP6FwCGG CTC GG CT CT TT TA AT G244FwCTC AAA TGC AAC GTA AGT AC AAA328RevAGT TCT TCC ATT TGT AG GGA AGC193VP7FwCTG TCA ATT CGA TAC TGC ACT TTG TAT AA133FwCTG TGA GAA AGA ATT CCA CAC CA240VP7FwCTG TGA GAA AGA ATT CGA AAT CGA ACT CCA ATT TG AAA TTG CAC260RevCCA TAT AAA ATA CAC GAA AAT CGC CAA196NSP1FwGGG AAA AGA TAA ACA CAT TG GAA ATC GC260RevCTA TAT GAA AGA CCAAA CCAA CAA TT GG AAA TTG GAA ATT GGA110FwGGG AAA CAA AGA CCAAT CTG AGC GA110FwGGG AAA CAA AGA CCAAT CTG CAA CGA151RevCTA GGG ATA GAA GAA GAA GAA TTA CCCA CGA231RevCTA ATT CAG CTC TG CAT CTT GCAA231RevCTA ATT CAG CTC TG CAAA ACGA GAA231RevCTA ATT CAG CTC TAT CAA CCC TT GCA237RevCCT ATT TC ACT CTC ACT GCA CTT GAA TTA CCA237RevCCT ATT TC CT AT AGA ATT CGC CAA CCAA231RevCCT ATT TC CT AT CAG CTC CCAA236RevCCT ATT TC CT AAG TCA TTG CAA237RevCCT ATT TC CT CT AGA CTC CT GCA CT GA CT CAA237RevCCT ATT TC CT CT AGA TTA CAA TTG CAA CTC CAA237RevCCT ATT TC CT CT
RevCTA TTA TCT TAT TCG AGA GAG GCT TTG TA330RevGGA GAG AAC GTC AGT ACC AAA TAA TTT CC376RevGGT TTC ACG TCC GAG TAT TTG CCA CCA CCA439VP6FwCGC TCG GC CT CGT GTT TTA AT C244FwCTC AAA TGC AAC CGA ACC GG AGC193VP7RevAGT TGT TCC ATT TCT ACG GGA AGC193VP7FwCTG TGA GAA AGC ATT GC ACT TTG TTT TAA A133FwCTG TGA GAA AAC ATT CGA CC CA CC ACT TTG TTT ATA A133FwCTG TGA GAA AAC ATT CAC AGC CA247RevCCA TAT AAA CAC GAA ACG CAA ATC GC260RevCCA TAT AAA CAC GAA ACA GAT GG GG AA196NSP1FwGGG AAA AGA TAA ACA ACT TGG AGA AGC AGC ATT TTG AG161NSP1FwGGA AAC AGA AGA AGA ACA ACA ACT GC AG151RevCTA GG AAA CGA AAC AGA AGA AGA ACA ACA ACT GC A231RevCTG AGA AAC GAA AACA AGA AGA ATTA CCA231RevCTG ATT CT CAT ATT CAG AGA AGA AGA ATTA CCA231RevCTA ATT CT CAT CT GG GT AT CT CT GG AGA ATTA234NSP3FwCCA AGC GAT ACA ATTA ACA CT CT GGA GTA278NSP3FwCCA AGT GATT CT AGA TTC TCA ATC ATC AGA TCC203RevCCA ATT ACA GATT TTA ACA TCT TCA ATC ATC AGA TCC203RevCCA ATT ACA ATT CT CCA AGT TCC203RevCCA ATT ACA ATT CT CT AGA TCC203RevCCA ATT TTC CTT AGA TCC TCT AGA TCC203RevCCA ATT TTC CTT AGA TCC TCT AGA TCC203RevCCA ATT TTC CTT AGA
RevGGA GAG AAC GTC AGT ACC AAA TAA TTT CC376RevGGT TTC ACG TCC GAA TAT TCG CCA CCA439VP6FwCAG CTC CGG CGT CGT TTT TAA TG244FwCTC AAA TGC AAC CGA CAC TAT CA328RevAGT TGT TCC ATT CGA TAC TGC AGC193VP7FwCTG TAA ATG CAAC GGA AGC193FwCTG TAA ATG CAAC AGC CAC CT TTG TTT ATA A133FwCTG TGA GAA AAG ATT CAT CAC ACC CA247RevCCA TAT AAA CAC GAA CAC TTT GAA ATC GC260RevTTT CAT ATG TAA ATC CCC TGA ACG AA196NSP1FwGGG AAA AGA TAA ACA ACT TGG AGT GA110NSP2FwCTG GGG ATA GAT TTT CAT ATG TGA ATC CGA151RevCGA GAA AGA AGA AGA AGA AGA ATA ACA GGA151RevCGG GAA AGA AGA AGA AGA AGA TATA CCA231RevCTG AGG AAA GGA AAG GAA AGA ATTA CCA231RevCCT ATT TC CT GA GA GA ATA CAT GG CCC TTG C237RevCCT ATT TC CAT CT GC AGA TTA CAT GA ATTA CAA278RevCCA AGT AGA CTT AGA CTC CCA168RevCCA GA GA ATT ATC GA TTG ATG ATA CAA224RevCCA ATT ATT CAA CTG GAA TTA ATT GAA224RevCCA ATT ATT CCA ATT TTC CTC AGT TTG ATG AA224RevCCA ATT ATT CCA ATT TTC CTC AGG TCC203RevCCA ATT ATT CCAA TTG CCA CCT TTG AGA245RevCCA ATT AT CCA ATT TTC CTC AGT CCA245RevCCA ATT AT CCA ATT TC CTC CAA CTCA245RevCCA ATT AT CCA ATT TC CTC CAA TTG CAA
RevGGT TTC ACG TCC GAA TAT TCG CCA CCA439VP6FwCAG CTC CGG CGT CGT TTT TAA TG244FwCTC AAA TGC AAC CGA ACG CAG CAG TAT CA328RevACT TGT TCC ATT TGT ACG GAA AGC193VP7FwCTG TCA ATT CGA TAC TGC ACT TTG TTT ATA A133FwGTG TGA GAA AAG ATT CAT CAC AGC CA247RevCCA TAT AAA CAC GAA CAT TTT GAA ATC GC260RevCCA TAT AAA CAC GAA CAT TTT GAA ATC GC260RevCTT CAT ATG TAA ATC CCC TGA ACG AA196NSP1FwGGG AAA AGA TAA ACA ACT TGG AGT GA110FwATC GAA GAA AGA TAA ACA CGA151RevGGG AAA CAC AAC CAT TTT CT TC TC161NSP2FwCTG GGG ATA CACA ACG AAC GCA ACG AA231RevCTT ATT CAG TCT CT ATC AGC CCC TTG C237RevCCT ATT TC AGC TCT ATC AGC CCC TTG CA278NSP3FwCAA GGA ATT ATC AGA TCT CG AG TGA278RevCCA ATT ATC GAC ATT TTC AGT CCA ATT TG AA TTG CAA224RevCCA ATT ATC GAC ATT TTC CTA ATTG CAA TTG CAA231RevCCA ATT ATC GAC ATT TTC CTC AGT CC CA168FwCAG GGT TA GAT TCT CTT ATA GAA TCT GAA224RevCCA ATT ATC GAC ATT TTC CTC AGG TCC203RevCCA ATT ATC GAC ATT TTT CTC AGG TCC203RevCCA ATT ATC GAC ATT TTC CTT ATT CAG TCC203RevCCA ATT ATC GAC ATT TTC CTT ATA CGAC TCT TTT CTT A245RevCCA ATT ATC CAC ATT TTC CTT ATT CCTC CAG TCC203Re
VP6FwCAG CTC CGG CGT CGT TTT TAA TG244FwCTC AAA TGC AAC CGA AGC TAT CA328RevAGT TGT TCC ATT TGT ACG GGA AGC193VP7FwCTG TCA ATT CGA TAC TGC ACT TTG TTT ATA A133FwGTG TGA GAA AAG ATT CAT CGC CAG247RevCCA TAT AAA CAC GAA CAT TTT GAA ATC GC260RevTTT CAT ATG TAA ATC CCC TGA ACG AA196NSP1FwGGG AAA AGA TAA ACA CAT TGG ACT GA110FwGGG AAA GAA GAA GAA ACA CAT TGG ACT GA110FwGGA AAC GAA AGC AAA CAC TTT CT CT161NSP2FwCTG GGG ATA GAA AGA AGG AGA AGA CAG AGA231FwCCA AGG AAC AAA GAG AGA AGA GAG AAA TTA CCA231RevCCT AAT TTC AGC TCT ATC GGG CCT TG C237RevGTC ATT TCT CTG CAT CG GA CT CGA248NSP3FwCCA AGC AAT CG TTT ATA CAT TTG AA224RevCCA ATT ATC GAC ATT TTC CTC AAG TCC203RevCCA ATT ATC GAC ATT TTC CTC AGT CC203RevCCA ATT ATC GAC ATT TTC CTC AGT CC203RevCCA ATT ATC CAC CTC ATTTC CTC AGT CC203RevCCA ATT ATC CAC CTC ATTTC CTC AGT CC203RevCCA ATT ATC CAC CTC CTC ATTTC CTC AGT CC203RevCCA ATT ATC CAC CTC CC203RevCCA ATT TTC CTT AG ATTTC TTT CTC AGT CC203RevCCA ATT ATC CAC CTC ATTTC CTC AGT CC203RevCCA ATT ATTC CAC CTC CC203RevCCA ATT ATT CCAC CTC CC203Rev<
FwCTC AAA TGC AAC CGA CAG TAT CA328RevAGT TGT TCC ATT TGT ACG GGA AGC193VP7FwCTG TCA ATT CGA TAC TGC ACT TTG TTT ATA A133FwGTG TGA GAA AAG ATT CAT CAC AGC CA247RevCCA TAT AAA CAC GAA CAT TTT GAA ATC GC260RevTTT CAT ATG TAA ATC CCC TGA ACG ACA196NSP1FwGGG AAA AGA TAA ACA CAT CGA CGA CAT TTT GAA151RevCCA GAA AGA CAA GAA ACA ACA ACA TCT TC TT CT161NSP2FwCTG GGG AAA AGA AAG ATA ACA ACA TT TTA CAA TTC GCA231RevCCA AAT TTC AGC TCT ATG CAA ATG CAG CAA231RevGTC ATT TC AGT CTC AGG CGA AAA TTA CCA231RevCCA ACT ATT CA CTG CAG CAA TTA CCA231RevGTC ATT CTC ATG CAT CTG GAG TAA278NSP3FwCCA CAG CATT AGT CTC CAA CAT TTG AA224RevCCA ATT ATC GAC ATT TTC CAA TTG AAA TCA TTG AA224RevCCA ATT ATC GAC ATT TTC CTC AAG TCC203RevCCA ATT ATC GAC ATT TTT CTC AAG TCC203RevCCA ATT ATC GAC ATT TTT CTC AAG TCC203RevCCA ATT ATC GAC ATT CTC TTT CTTA AG TAA TCA TTG AA224RevCCA ATT ATC GAC ATT TTT CTC AAG TCC203RevCCA ATT ATC GAC ATT TTT CTT CTT CTT AAG TAA TCA TTG AA224RevCCA ATT ATC GAC ATT TTT CTT CTT CTA AG TAG TCC203RevCCA ATT ATC GAC ATT TTT CTT CTT CTA AG TCC203RevCCA ATT ATC GAC ATT TTT CTT CTT AAG TAG TCC203RevCCA ATT ATC GAC ATT T
RevAGT TGT TCC ATT TGT ACG GGA AGC193VP7FwCTG TCA ATT CGA ATC TGC ACT TTG TTT ATA A133FwGTG TGA GA AAGA ATT CAT CAC AGC CA247RevCCA TAT AAA CAC GAA CAT TTT GAA ATC GC260RevTTT CAT ATG TAA ATC CCC TGA ACG AA196NSP1FwGGG AAA GCA ACA CAC ACT GG ACT GA196FwGGG AAA GCA ACA CAA ACA CAC ACA CGA151FwGGA AAA GCA ACC AAA CAC ACC ACC AAA151FwCTG GGG ATA GAA ACG ACC ATC TTT CTC TC161NSP2FwCTG GGG ATA GAA AGA GAA AGA GAA ATTA CCA231RevCCT AAT TTC AGC TCT ATC GCC TTG CT237RevGTC ATT CTC ATT CAG TCT GGA GTA278RevCCA GTA CGA TTC ATT CAG TCT CAAT TTG AAA224RevCCA ATT ATC GAC ATT TTC CTC AGT CTC AGT CCC203RevGTC ATT TCC GCA ATT TTC CTT CAG TTC AGT CCC203RevGTC ATT TCC GCA CTT TTT CTT CTC AGT CCA245RevGTC ATT TCC GCA TTT CTC TTT CTT ACA TTG CAA245RevCCA ATT ATC GAC ATT TTC CTC AGT CCC203RevGTC ATT TCC GCA TTT CTC TTT CGAAT TCC TTT CACA245RevGTC ATT TCC GCA TTTT CTC TTT CTC AGT CCC203RevGTC ATT TCC GCAT TTT CTT CCC101RevGTC ATT TCC GCAT TTT CTT CCC102RevCCA ATT ATC CCC TTT CTC TTTT CTC AGT CCC203RevGTC ATT TCC GCAT CTT CTC TTTT CTC CCC203RevGTC ATT TCC CTC TTTT CTC CCC203RevGTC ATT TCC CTC TTTTT C
VP7FwCTG TCA ATT CGA TAC TGC ACT TTG TTT ATA A133FwGTG TGA GAA AAG ATT CAT CAC AGC CA247RevCCA TAT AAA CAC GAA CAT TTT GAA ATC GC260RevTTT CAT ATG TAA ATC CCC TGA ACG AA196NSP1FwGGG AAA AGA TAA ACA CAC ACA CT GA CT GA110FwGGA AAG AGA GCA ACC ACT CTT CT CT161NSP2FwCTG GGG ATA GAT ACA GAA ACG ACG CAA174FwCCA GGA AAC GAA ACG ACG AAC TG GCA174FwCTG GGG ATA GAT ATTT CAA TGT GCA174RevCCT GGG ATA GAT ACT AGA GCA ACG AAG TAA CCA231RevCTC AAT TTC AGC TCT ATC AGC CCT TG C237RevCTC ATT CTC CTA CTG CAT CT GGA GTA278RevCCA AGT ACT CT ATA TC AGT CT CAA168FwCAA GGA ACT TTT CTA TAG TAA TCA TTG AA224RevCCA ATT ATC GAC ATT TTC CTC AGT CTC CAA CTG CC203RevGT CATT CCTC TTT GG AAT TCT TTT CTT AT245RevCCA ATT ATC CAC CTT CTT CTC CTA CTA CTG CAA CTC100RevCCA ATT ATC CAC ATT TTC CTC CAG TTC CTA CTG CAA168FwCAC TGA TTC CTT TG AAT CCAT TTG AA224RevCCA ATT ATC GAC ATT TTC CTC AAG TTC CTT CTA AGT CCC203RevCCA ATT ATC CAC ATT TTC CTC CAG TTC CTA CTG CAA245RevCCA ATT TC CTT CTT CTT CTA CTC CCA100
FwGTG TGA GAA AAG ATT CAT CAC AGC CA247RevCCA TAT AAA CAC GAA CAT TTT GAA ATC GC260RevTTT CAT ATG TAA ATC CCC TGA ACG AA196NSP1FwGGG AAA AGA TAA ACA ACT TGG AGT GA110FwATC GAA GAA GC AAG CAA ACA CGA151RevGGA AAC AAA GCA ACC ATC TTT CTC TC161NSP2FwCTG GGG ATA GAT AGA AGA AGA ATA CCA231FwCTG AGG AAC AGA AGA AGA AGA GG AAA TTA CCA231RevCTC ATT TTC AGT CTT ATC GG CTT GC237RevCTC ATT CTC AGT CTC ATC GG GAT ACT CT GG AGT ACT CTG GA168FwCAC AGA GTT AGA TTC ATG GCT CCA168FwCAG TGA TTC GAC ATT TTC AGT CTC AGT CTC GAG TTC203RevCCA ATT ATC GAC ATT TTC CTC AAG TTCC TA ATT CTC AGT CTC CTA ATT CAGT CTC AGT CTC CTA ATT CAGT CTC AGT CT
RevCCA TAT AAA CAC GAA CAT TTT GAA ATC GC260RevTTT CAT ATG TAA ATC CCC TGA ACG AA196NSP1FwGGG AAA AGA TAA ACA CT TGG AGT GA110FwATC GAA GCA AGC AAA ACA CGA151RevGGA AAC AAA GCA ACC ATC TTT CTC TC161NSP2FwCTG GGG ATA GAA TTA CAC AGG AAA TTA CCA231RevCCT AAT TTC AGC TCT ATC AGC CCC TTG C237RevGTC ATT CC AGC CTT ATC AGC GCT AGC TTA CCA231RevGTC ATT CTC CTC AGT CT GG AGT CC GA GTA278NSP3FwCCA GAC GTT AGA TTC ATG GCT CCA168FwCAA GCA ATT ATC GAC ATT TTC AGT CC CAA203RevCCA ATT ATC GAC ATT TTC CTC AAG TCC203RevGTC ATT CC CTT CTC TTG GAAT TCT TTT CTT A245RevCCA ATT ATC GAC ATT TTC CTC CAG TCC203RevCCA ATT CC CTC TTT GG AAT TCT TTT CTT A245RevCCA ATT CC CTC TTT CTC TTG CCAC100
RevTTT CAT ATG TAA ATC CCC TGA ACG AA196NSP1FwGGG AAA AGA TAA ACA ACT TGG AGT GA110FwATC GAA GAA GCA AGC AAC ACG CAA151RevGGA AAC AAA GCA ACC ATC TTT CTC TC161NSP2FwCTG GGG ATA GAT ACA AGA GGA AGT CAA TTA CCA231RevCCT AAT TTC AGT CTC AGC CCC TTG C237RevGTC ATT CTC ATG CAT CTT CAG GG ATA278NSP3FwCCA GAC GTT AGA TTC ATG GCT CCA168FwCAG TGA TCG CTT CTC TA TAG TAA TCA TTG AA224RevCCT ATT ATC CAC ATT TTC CTC AGG TCC203RevGTC ATT TC GCA CTT TTG CAA TCT TTC CTT A245RevCCT ATT TTC CTT CTT CTT CTT A245
NSP1FwGGG AAA AGA TAA ACA ACT TGG AGT GA110FwATC GAA GAA GCA AGC AAG CGA151RevGGA AAC AAA GCA ACC ATC TTT CTC TC161NSP2FwCTG GGG ATA GAT TTT TAT CAA TGT GCA174FwCAA GGA AAC AAA GGA AGG AAA TTA CCA231RevCCT AAT TTC AGC TCT ATC AGC CCC TTG C237RevGTC ATT CTC CTG CAT CTG GGA GTA278NSP3FwCCA GAC GTT AGA TTC ATG GCT CCA168FwCAG TGA TCG CTT CTA TAG TAA TCA TTG AA224RevCCC ATT ATC GAC ATT TTC CTC AAG TCC203RevGGT CAT TTC CTT TGG AAT TCT TTT CTT A245RevCCA ATT ATC GCA CATT TTC CTT CACA130
FwATC GAA GAA GCA AGC AAA ACA CGA151RevGGA AAC AAA GCA ACC ATC TTT CTC TC161NSP2FwCTG GGG ATA GAT ATT TAT CAA TGT GCA174FwCAA GGA AAC AGA AAG AGG AAA TTA CCA231RevCCT AAT TTC AGC TCT ATC AGC CCC TTG C237RevGTC ATT CTC CTA CTG GAG GTA278NSP3FwCCA GGA ACG TTT AT CAA TCA TTG AA224RevCCA ATT ATC GAC ATT TTC CTC AGT CCC203RevGTC ATT TC CTT TG GAA TTC TTT CTT A245RevCCA ATT TTC CTT AGG CAT CTT TAT CTT AG245RevCGT ATT TTC CTT AGT CAGT TCT TAT CTT AG245RevCGT ATT TTC CTT TGG AAT TCT TTT CTT A245RevCGT CAT TTC CTT TGG CAT TTC CTT AGT CAGT C
RevGGA AAC AAA GCA ACC ATC TTT CTC TC161NSP2FwCTG GGG ATA GAT TTT TAT CAA TGT GCA174FwCAA GGA AAC AGA AGA AGA GGA AAT TA CCA231RevCCT AAT TTC AGC TCT ATC AGC CCT TG C237RevGTC ATT CTC CTA CTG CAT CTT GGA GTA278NSP3FwCCA GGA TCG TT CAT TG TAG TCA CTG CAA168FwCAA TGA TGC GCT CTT ATA GTAA TCA TTG AA224RevCCA ATT ATC GAC ATT TTC CTC AGG TCC203RevGGT CAT TTC CTT CTT GG AAT TCT TTT CTT A245RevCCA ATT ATC CAA TTC CTT CTT CTT AG245RevCCA ATT CCATT CTT CTT CTT CTT AG245RevCCA ATT CCATT CTT CTT CTT CTT AG245RevCCA ATT CCATT CTT CTT CTT CTT AG245RevCCA ATT TCC CTT TGG AAT TCT TTT CTT A245RevCCA ATT CCATT CTT CTT CTT CTT AG245RevCCA ATT TCC CTT TGG AAT TCT TTT CTT A245RevCCA ATT TCC CTT CTT CTT CTT CTT AG TCA CTT CCAL245RevCCA ATT TCC CTT CTT CTT CTT CTT AG245RevCCA ATT TCC CTT CTT CTT CTT CTT AG245RevCCA ATT TCC CTT CTT CTT CTT CTT CTT CTT
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 AGT CTA CGG CAT CT GGA GTA       278         NSP3       Fw       CCA GAC GTT AGA TTC AGC CCC CAA       168         Fw       CCA ATT ATC GAC ATT TTC CTC AAG TCC AGA TCC TG 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         NDP4       Fw       CCA CGA CAT TTC CTT TGG CAT TCC TT A       245
FwCAA GGA AAC AGA AAG AGG AAA TTA CCA231RevCCT AAT TTC AGC TCT ATC AGC CCC TTG C237RevGTC ATT CTC CTA CTG CAT CCT GGA GTA278NSP3FwCCA GAC GTT AGA TTC ATG GCT CCA168FwCAG TGA TCG CTT CTA TAG TAA TCA TTG AA224RevCCA ATT ATC GAC ATT TTC CTC AAG TCC203RevGGT CAT TTC CTT TGG AAT TCT TTT CTT A245RevCCA CTA TTC CCAT CTT CTA CTC CCA100
RevCCT AAT TTC AGC TCT ATC AGC CCC TTG C237RevGTC ATT CTC CTA CTG CAT CCT GGA GTA278NSP3FwCCA GAC GTT AGA TTC ATG GCT CCA168FwCAG TGA TCG CTT CTA TAG TAA TCA TTG AA224RevCCA ATT ATC GAC ATT TTC CTC AAG TCC203RevGGT CAT TTC CTT TGG AAT TCT TTT CTT A245
RevGTC ATT CTC CTA CTG CAT CCT GGA GTA278NSP3FwCCA GAC GTT AGA TTC ATG GCT CCA168FwCAG TGA TCG CTT CTA TAG TAA TCA TTG AA224RevCCA ATT ATC GAC ATT TTC CTC AAG TCC203RevGGT CAT TTC CTT TGG AAT TCT TTT CTT A245
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
FwCAG TGA TCG CTT CTA TAG TAA TCA TTG AA224RevCCA ATT ATC GAC ATT TTC CTC AAG TCC203RevGGT CAT TTC CTT TGG AAT TCT TTT CTT A245CCACCA ATT ATC GCA CAT CTT TCT CTA245
Rev     CCA ATT ATC GAC ATT TTC CTC AAG TCC     203       Rev     GGT CAT TTC CTT TGG AAT TCT TTT CTT A     245
Rev     GGT CAT TTC CTT TGG AAT TCT TTT CTT A     245       NICDA     Example 1     120
NSP4 FW TAA AGA GGA CAT CAT GTA ACT CCA GGA T20
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 C
NSP5 Fw CTC TCT CCA AAA TTA ATC CTT CCA GAA AA 427
Rev CAT GGA GGA GCG TIT TCT TCT GTG GTG TA
Screening PCR VP6 Fw GGA TICTICA AA TAT CTC CAA C 643 (1st round)
Rev GGA ACT TGA ATA AAA CCT GG
Ew CGATTA CAA CAT IGC TTC 338 (2nd round)
Rev GTT CCA TTC TAG CTG TAT CA
d There eligenerical the test of DC2 med and DC2 med and the test of the Designment (2002)

reverse transcribed and amplified using QIAGEN One-Step RT-PCR Kit (Qiagen) in a 25  $\mu$ L 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  $\mu$ L of the first round PCR products were amplified with inner primers for 35 cycles under the following conditions: initial denaturation (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 <sup>a</sup>	Assignment based on the main gene product	Positions of s codons	start and stop	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					

<sup>a</sup> 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

Co	ompari	ison o	f the	genome	size	and	the	coding	potentia	l of	differ	ent l	RV	species
----	--------	--------	-------	--------	------	-----	-----	--------	----------	------	--------	-------	----	---------

	Rotav Wa	rus A, Rotavirus A, 02V0002G3		a Rotavirus A, 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 <sup>a</sup>	
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	3305	VP1	3511	VP1	3309	VP1	3274	VP1	3296	VP1	3526	VP1	3538	VP1	3518	VP1	3533	VP1	
	0.54.5	(1088)	0700	(1089)	20.47	(1160)	0700	(1090)	0001	(1079)	0700	(1086)	2011	(1160)	2000	(1167)	2002	(1162)	2010	(1168)	
2	2717	(800)	2732	(805)	2847	VP2 (024)	2736	VP2	2801	VP2 (012)	2769	VP2 (004)	3014	VP2 (001)	2969	VP2 (072)	3002	(082)	3010	VP2	
3	2591	(890) VP3	2583	(895) VP3	2341	(954) VP3	2283	(004) VP3	2366	(915) VP4	2246	(904) VP4	2364	(991) VP4	2512	(975) VP4	2371	(962) VP4	2512	(980) VP4	
5	2551	(835)	2505	(829)	2541	(763)	2205	(693)	2500	(777)	2240	(738)	2504	(772)	2512	(823)	2571	(777)	2312	(826)	
4	2359	VP4	2354	VP4	2306	VP4	2166	VP4	2104	VP3	2174	VP3	2352	VP3	2204	VP3	2161	VP3	2200	VP3	
		(775)		(770)		(750)		(744)		(685)		(694)		(768)		(719)		(701)		(715)	
5	1567	NSP1	2122	NSP1	1276	NSP1-1	1353	VP6	1872	NSP1	1791	NSP1	1295	NSP1-1	1307	NSP1	1485	NSP1-1	1322	NSP1	
		(486)		(577)		(107)		(395)		(574)		(547)		(106)		(395)		(79)		(401)	
						NSP1-2								NSP1-2				NSP1-2			
						(321) NGD1 2								(324)				(390)			
						(65)															
6	1356	VP6	1348	VP6	1269	VP6	1350	NSP3	1353	VP6	1314	VP6	1267	VP6	1287	VP6	1278	VP6	1277	VP6	
		(397)		(397)		(391)		(402)		(398)		(396)		(391)		(396)		(395)		(395)	
																				ORF-X	
																				(223)	
7	1074	NSP3	1089	NSP3	1179	NSP3	1270	VP6	1242	NSP3	1309	NSP3	1052	NSP3	1004	NSP3	1018	NSP2	1108	NSP3	
0	1000	(310)	1000	(304)	1007	(347)	1000	(394)	1020	(370)	1000	(370)	1012	(300)	022	(297)	054	(301)	1017	(331)	
δ	1062	(226)	1066	(220)	1007	NSP2 (201)	1063	(222)	1026	(210)	1068	(219)	1012	(282)	932	(362)	954	NSP3 (272)	1017	(200)	
9	1059	(320) NSP2	1042	(329) NSP2	814	(301) VP7	1037	(332) NSP2	1025	(310) VP7	990	(516) VP7	825	(202) VP7	820	(202) VP7	858	(275) VP7	793	(299) VP7	
5	1055	(317)	1042	(315)	014	(249)	1057	(312)	1025	(316)	550	(295)	025	(247)	020	(258)	050	(268)	155	(245)	
10	750	NSP4	724	NSP4	751	NSP4	730	NSP5	765	NSP4	706	NSP5	801	NSP4	739	NSP4	751	NSP4	743	NSP4	
		(175)		(168)		(219)		(212)		(127)		(218)		(187)		(213)		(219)		(210)	
										ORF2											
										(93)											
11	664	NSP5	699	NSP5	631	NSP5	615	NSP4	672	NSP5	678	NSP4	678	NSP5	649	NSP5	593	NSP5	620	NSP5	
		(197) NGDC		(208)		(170)		(150)		(195)		(169)		(181)		(176)		(157)		(165) OBE V	
		01210 (92)																		(73)	
Total	18.50	1	19.06	4	17.93	2	17.91	2	18.50	D	18.34	1	18.18	6	17.96	1	17.98	9	18.13	5	
	12,20		12,50		,		,		,	-	,.		,.0	-	,50		,	-	,	-	

<sup>a</sup> Abbreviated name of BO4351/Ms/2014.

24

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







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

## 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														
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	<	39	21	30	<	31	<	31	<	39	18	47	34	44	26
		10				10		10		10						
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

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



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.



RVA RVB RVC RVD RVF RVG RVH RVI

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 DIA-MOND. 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 largescale 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 f
  ür 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.