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Research paper

Identification of group A rotaviruses from Zambian fruit bats provides evidence for long-distance dispersal events in Africa

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ABSTRACT

Group A rotavirus (RVA) is a major cause of diarrhea in children worldwide. Although RVA infects many animals, little is known about RVA in bats. The present study investigated the genetic diversity of RVA in Zambian bats. We identified RVA from two straw-colored fruit bats (*Eidolon helvum*) and an Egyptian fruit bat (*Rousettus aegyptiacus*), and analyzed the genome sequences of these strains. Genome segments of the RVA strains from Zambian *E. helvum* showed 97%–99% nucleotide sequence identity with those of other RVA strains from *E. helvum* in Cameroon, which is 2800 km from the sampling locations. These findings suggest that migratory straw-colored fruit bat species, distributed across sub-Saharan Africa, have the potential to disseminate RVA across long distances. By contrast, the RVA strain from Zambian *R. aegyptiacus* carried highly divergent *NSP2* and *NSP4* genes, leading us to propose novel genotypes N21 and E27, respectively. Notably, this RVA strain also shared the same genotype for *VP6* and *NSP3* with the RVA strains from Zambian *E. helvum*, suggesting interspecies transmission and genetic reassortment may have occurred between these two bat species in the past. Our study has important implications for RVA dispersal in bat populations, and expands our knowledge of the ecology, diversity and evolutionary relationships of RVA.

1. Introduction

Rotavirus is a major causative agent of gastroenteritis in children under five, with > 120,000 cases of diarrheal death annually, worldwide (Clark et al., 2017). Among nine species of rotavirus (groups A to I), group A rotavirus (RVA) is the major species and the most well studied to date. RVA has a genome of 11 segments of double-stranded RNA, which encode the viral structural proteins (VP1-4, VP6 and VP7) and the non-structural proteins (NSP1-6). The current nomenclature system of RVA defines the genotype as: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx for the VP7-, VP4-, VP6-, VP1-, VP2-, VP3-, NSP1-, NSP2-, NSP3-, NSP4- and NSP5/6-encoding genes, respectively (Matthijnssens et al., 2008, 2011a). Based on the genome sequence, all RVA isolates are classified into genotypes in accordance with the recommendations of the Rotavirus Classification Working Group (RCWG) to ensure uniformity (Matthijnssens et al., 2008). This classification system has been widely adopted and has greatly facilitated the analysis of RVA sequence data, which has uncovered high genetic diversity and proposed new genotypes (Esona et al., 2018; He et al., 2017; Ianiro et al., 2017; Li et al., 2016; Rojas et al., 2016, 2017; Yinda et al., 2016).

Bats harbor numerous pathogens and act as reservoir hosts of high-consequence zoonotic viruses (Hayman, 2016; Olival et al., 2017). A limited number of studies have reported on RVA from frugivorous bats: *Eidolon helvum* in Kenya and Cameroon (Esona et al., 2010; Yinda et al.,

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2016), *Rousettus aegyptiacus* in Kenya (Waruhiu et al., 2017) and *Rousettus leschenaultii* in China (He et al., 2017), and insectivorous bats: *Molossus molossus* in Brazil (Asano et al., 2016), *Rhinolophus simulator* in Zambia (Sasaki et al., 2016), *Taphozous mauritianus* in Kenya (Waruhiu et al., 2017), and *Rhinolophus hipposideros*, *Aselliscus stoliczkanus*, *Scotophilus kuhlii*, *Hipposideros pomona* and *Taphozous melanopogon* in China (He et al., 2013, 2017; Xia et al., 2014). Genetic characterization of bat RVAs has led to discoveries of new RVA genotypes. In addition, these studies revealed that bat RVAs not only carry unique genotypes exclusively observed in bats, but also share some genome segments with RVAs derived from humans and other mammals, indicative of interspecies transmission and the zoonotic potential of bat-borne RVA (Esona et al., 2010; He et al., 2017; Sasaki et al., 2016). Although the sporadic detection of RVA from bats worldwide has demonstrated that RVA infection can occur in some bat species, thus far, the genotypic tropism(s) and transmission cycle of RVA in bat populations are poorly understood.

Previously, we reported RVA strain LUS12-14 from the insectivorous horseshoe bat species, *R. simulator*, in Zambia (Sasaki et al., 2016). In the present study, we screened insectivorous and frugivorous bat species in Zambia to investigate the prevalence of RVA infection and also to determine host species susceptible to RVA infection. Three RVA strains were newly identified from the fruit bats, *E. helvum* and *R. aegyptiacus*. Our findings have important implications for RVA dissemination across long distances in African fruit bats and provide evidence of interspecies transmission and genetic reassortment events among African bat RVAs.

2. Materials and methods

2.1. Sample collection and ethics statement

From 2014 to 2015, 60 frugivorous and 40 insectivorous bat species were captured at five different locations in Zambia, with permission from the Department of National Parks and Wildlife (formerly the Zambia Wildlife Authority), Ministry of Tourism and Arts (Act No. 12 of 1998). Spleen, liver, kidney and colon tissues were collected through dissection. Bats were speciated based on morphology and sequencing of ribosomal RNA and cytochrome *b* loci, as previously described (Sasaki et al., 2012). Sample information is summarized in Table 1.

2.2. Nested RT-PCR screening for RVA

Total RNA was extracted from bat colon tissue using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For nested RT-PCR screening, cDNA was synthesized using random hexamers and SuperScript IV Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA), and subjected to nested PCR amplification employing the Tks Gflex DNA polymerase (Takara Bio, Kusatsu, Japan) and oligonucleotide primers targeting RVA VP7 as follows: RotexoF (5′- MDCGGWTAGMYYBTTTWAATG -3′) and RotexoR (5′- CCCATNGMDATCCAYTTRTT -3′) for the 1st round PCR, and RotinF (5′- TAGCYYBTTTTRATGTATGGKAT -3′) and RotinR (5′- TCCATNGGRTTRCAHARCC -3′) for the 2nd round PCR (Li et al., 2016). The thermocycling conditions were: 1 cycle of 94 °C for 2 min followed by 35 cycles of 98 °C for 10 s, 46 °C (1st PCR) or 50 °C (2nd PCR) for 15 s and 68 °C for 30 s. Amplicons were purified with the MonoFas DNA Purification Kit I (GL Sciences, Tokyo, Japan) and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Thermo Fisher Scientific).

Table 1
Sample information and RT-PCR screening results for rotavirus.

Bat species	Location	RT-PCR positive/total
Fruit bats		
Eidolon helvum	Ndola	1/10
Eidolon helvum	Kasanka national park	1/10
Epomophorus crypturus	Monze	0/20
Rousettus aegyptiacus	Lusaka	1/20
Insectivorous bats		
Hipposideros gigas	Lusaka	0/10
Miniopterus schreibersii	Lusaka	0/10
Nycteris sp.	Livingstone	0/20

2.3. Amplification and sequencing of RVA genome segments

Each genome segment was separately amplified by a nested RT-PCR strategy. After denaturation at 95 °C for 5 min, RNA samples were reverse transcribed with SuperScript IV Reverse Transcriptase and specific primer sets targeting the 5′ and 3′ ends of each of the 11 RVA genome segments, referred to as exoF or exoR, as described previously (Li et al., 2016). The 1st round PCR was performed with Tks Gflex DNA polymerase and the gene-specific primer pairs that were used in the reverse transcription step. The 2nd round PCR was performed with Tks Gflex DNA polymerase and the inner primer set, referred to as inF or inR as described previously (Li et al., 2016). The PCR amplicons were sequenced as described above.

2.4. Assignment of RVA genotypes

Genotypes of the identified segments were determined using the online tool RotaC (http://rotac.regatools.be) or following the judgment of RCWG (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg) (Maes et al., 2009).

2.5. Phylogenetic analysis

Maximum likelihood phylogenetic trees with 500 bootstrap replicates were inferred from multiple nucleotide sequence alignments of full-length genes of RVA reference strains and bat RVAs using MEGA7 software (Kumar et al., 2016). For the maximum likelihood analyses, the GTR + G + I model for *VP1* and *VP6*, the GTR + G model for *VP7*, *NSP2* and *NSP3*, and the TN93 + G model for *NSP4* were employed based on the “Find best DNA/protein model” in the MEGA7 software.

2.6. Nucleotide sequence accession numbers

The determined RVA genome sequences were deposited in the DDBJ/EMBL/GenBank database under accession no. LC277159–LC277170.

3. Results

3.1. Detection of RVA VP7 genome segments in Zambian fruit bats

During 2014–2015, three frugivorous bat species (*E. helvum*, *Epomophorus crypturus*, *R. aegyptiacus*) and three insectivorous bat species (*Hipposideros gigas*, *Nycteris* sp., *Miniopterus schreibersii*) were captured in Zambia (Table 1). No bats showed signs of serious infection, including diarrhea. RNA was extracted from 100 bat colon samples and subjected to nested RT-PCR screening targeting the conserved *VP7* gene of RVA. The screening identified three *VP7* positive samples from Zambian fruit bats: strain ZFB14-52 from an adult male *E. helvum*, ZFB14-135 from an adult female *E. helvum* and ZFB14-126 from an adult female *R. aegyptiacus*. To determine the genotype, we attempted to amplify the near-complete sequence of the *VP7* gene and recovered it from ZFB14-52 and ZFB14-135, but not ZFB14-126.

3.2. Detection of RVA genome segments from VP7-positive bats

To further characterize the RVA strains detected in Zambian fruit

Table 2
Genotype constellations of African bat-borne RVA strains.

Strain name	Host	Location	Genotype										
			VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Bat-wt/ZMB/ZFB14-52/2014/G31P[x] [a]	***Eidolon helvum***	**Zambia**	**G31**	P[x]	**I22**	Rx	Cx	Mx	Ax	Nx	**T17**	Ex	Hx
RVA/Bat-wt/ZMB/ZFB14-135/2014/G31P[x]	***Eidolon helvum***	**Zambia**	**G31**	P[x]	**I22**	**R15**	Cx	Mx	Ax	Nx	**T17**	Ex	Hx
RVA/Bat-wt/ZMB/ZFB14-126/2014/GxP[x]	***Rousettus aegyptiacus***	**Zambia**	Gx	P[x]	**I22**	Rx	Cx	Mx	Ax	**N21**	**T17**	**E27**	Hx
RVA/Bat-wt/KEN/KE4852/07/2007/G25P[6]	*Eidolon helvum*	Kenya	G25	P[6]	I15	Rx	C8	Mx	Ax	N8	T11	E2	H10
RVA/Bat-wt/CMR/BatLi08/2014/G31P[42]	*Eidolon helvum*	Cameroon	G31	P[42]	I22	R15	C15	M14	A25	N15	T17	E22	H17
RVA/Bat-wt/CMR/BatLi09/2014/G30P[42]	*Eidolon helvum*	Cameroon	G30	P[42]	I22	R15	C15	M14	A25	N15	T17	E22	H17
RVA/Bat-wt/CMR/BatLi10/2014/G30P[42]	*Eidolon helvum*	Cameroon	G30	P[42]	I22	R15	C15	M14	A25	N15	T17	E22	H17
RVA/Bat-wt/CMR/BatLy17/2014/G30P[47]	*Eidolon helvum*	Cameroon	G30	P[47]	I22	R15	C15	M14	A25	N15	T17	E22	H17
RVA/Bat-wt/ZMB/LUS12–14/2012/G3P[3]	*Rhinolophus simulator*	Zambia	G3	P[3]	I3	R2	C2	M3	A9	N2	T3	E2	H3

[a] Strains and genotypes reported in the present study are shown in bold.

bats, we sought to identify the remaining 10 genome segments of RVA in the *VP7*-positive specimens. The genome segments were amplified by nested RT-PCR. All RT-PCR products were sequenced directly and multiple peaks were not observed in the sequencing electropherogram, suggesting each amplicon originated from a single RVA strain. We determined the sequences of *VP6* and *NSP3* from strain ZFB14-52, *VP6* and *NSP2-4* from strain ZFB14-126, and *VP1*, *VP6* and *NSP3* from strain ZFB14-135 (Table 2). Despite multiple attempts by RT-PCR, the sequences of the other RVA genome segments remain to be elucidated.

3.3. Sequence comparison and phylogenetic analysis of VP7, VP1, VP6 and NSP3

Genotype identification was performed employing the RotaC online tool, which indicated that *VP7* of ZFB14-52 and ZFB14-135 could be assigned to the G31 genotype (Table 2). The sequence of these *VP7* genome segments showed 98% nucleotide identity to RVA strain BatLi08, belonging to the G31 genotype, which was discovered previously from *E. helvum* in the South West region of Cameroon (Yinda et al., 2016). Phylogenetic analysis of *VP7* showed that ZFB14-52 and ZFB14-135 clustered with BatLi08 and were distantly related to BatLi09, BatLi10 and BatLy17 belonging to the G30 genotype (Fig. 1), which were also identified from *E. helvum* in Cameroon (Yinda et al., 2016).

The *VP1* of ZFB14-135 showed 94% nucleotide identity with that of BatLi08 belonging to the R15 genotype. The *VP6* of ZFB14-52, ZFB14-126 and ZFB14-135 showed 97%, 90% and 99% nucleotide identities with that of BatLi08 belonging to the I22 genotype, respectively. The *NSP3* of ZFB14-52, ZFB14-126 and ZFB14-135 showed 98%, 90% and 99% nucleotide identities with that of BatLi08 belonging to the T17 genotype, respectively. Phylogenetic analyses of these genome segments revealed that ZFB14-52, ZFB14-126 and ZFB14-135 formed a discrete cluster with Cameroonian bat RVAs (BatLi08, BatLi09, BatLi10 and BatLy17) and were clearly separable from other previously described bat RVAs (Fig. 1). Collectively, these results indicated that Zambian fruit bat RVAs harbor the same genotypes of VP1, VP6, VP7 and NSP3 as Cameroonian fruit bat RVAs and exhibit high nucleotide sequence identities with these genome segments.

3.4. Identification of novel NSP2 and NSP4 genotypes

The NSP2 and NSP4 genotypes of ZFB14–126 could not be determined by RotaC due to their nucleotide sequence divergence. BLAST search analyses indicated that both NSP2 and NSP4 of ZFB14-126 showed < 80% nucleotide sequence identity with all available RVA sequence data deposited in the DDBJ/EMBL/GenBank public databases. Therefore, these sequences were submitted to RCWG and were approved as new genotypes: N21 for NSP2 and E27 for NSP4 (RCWG, 2018). Phylogenetic analyses revealed that NSP2 of ZFB14-126 was distantly related to other RVAs and segregated in a different clade from the Cameroonian bat RVA N15 genotype (Fig. 2). Furthermore, NSP4 of ZFB14-126 was highly divergent from all other RVAs and represented a distinct lineage of NSP4 (Fig. 2). These findings indicate that RVA strain ZFB14-126 possessed discordant NSP2 and NSP4 gene segments when compared with other genome segments.

4. Discussion

E. helvum is distributed across sub-Saharan Africa and previous studies revealed that the mean migratory distance of *E. helvum* was 860 km with a range from 270 to 3000 km (Ossa et al., 2012; Richter and Cumming, 2008). Prior genetic studies revealed a panmictic population of *E. helvum* across continental Africa, suggesting that this bat species travels and interbreeds over long distances (Peel et al., 2013). In Zambia, over one million *E. helvum* roost from October to December (Peel et al., 2017). Previous reports have suggested that migration of *E. helvum* facilitates the introduction of viruses into the bat population, such as filoviruses, henipaviruses, lyssaviruses and coronaviruses (Drexler et al., 2012; Leopardi et al., 2016; Ogawa et al., 2015; Peel et al., 2013).

In this study, we identified bat RVA strains ZFB14–52 and ZFB14–135 from *E. helvum* in Zambia, which belong to genotypes G31 for *VP7*, R15 for *VP1*, I22 for *VP6*, and T17 for *NSP3*. These genotypes were initially identified from Cameroonian *E. helvum* by another research group who proposed that novel RVA genotype constellations exist in *E. helvum*, such as has been determined in humans and domesticated animals (Matthijnssens et al., 2011b; Matthijnssens and Van Ranst, 2012; Yinda et al., 2016). Our results support this view that certain RVA genotype constellations exist in this bat species. Interestingly, these Zambian bat RVA strains (ZFB14–52 and ZFB14–135) carried *VP7*, *VP6* and *NSP3* genome segments that shared 97%–99% nucleotide sequence identity with those of BatLi08 from *E. helvum* in Limbe, Cameroon, at least 2800 km apart from our sampling locations. Notably, it has been reported that RVA strain BatLy03 from Cameroonian *E. helvum* shared the same genotypes for *VP2*, *VP6*, *VP7*, *NSP2*, *NSP3* and *NSP5* as strain KE4852 from Kenyan *E. helvum* (Yinda et al., 2016). These findings suggest that the migration of *E. helvum* may have the potential to spread RVA across long distances and impact on the viral ecology.

Recent genetic analyses of bat RVAs have discovered new genotypes of this virus (Asano et al., 2016; Esona et al., 2010; He et al., 2017; Yinda et al., 2016). In this study, we identified the previously unrecognized genotypes N21 for *NSP2* and E27 for *NSP4* in RVA strain ZFB14–126 from *R. aegyptiacus* in Zambia. Both N21 and E27 were distinguished from other mammalian RVAs by long branch lengths in their phylogenies (Fig. 2). These results indicate that previously unrecognized genotypes are harbored by bats with unique evolutionary histories. In addition, ZFB14–126 shared the same I22 and T17 genotypes with ZFB14–52 and ZFB14–135 from *E. helvum* (Table 2), suggesting interspecies transmission and genetic reassortment may have

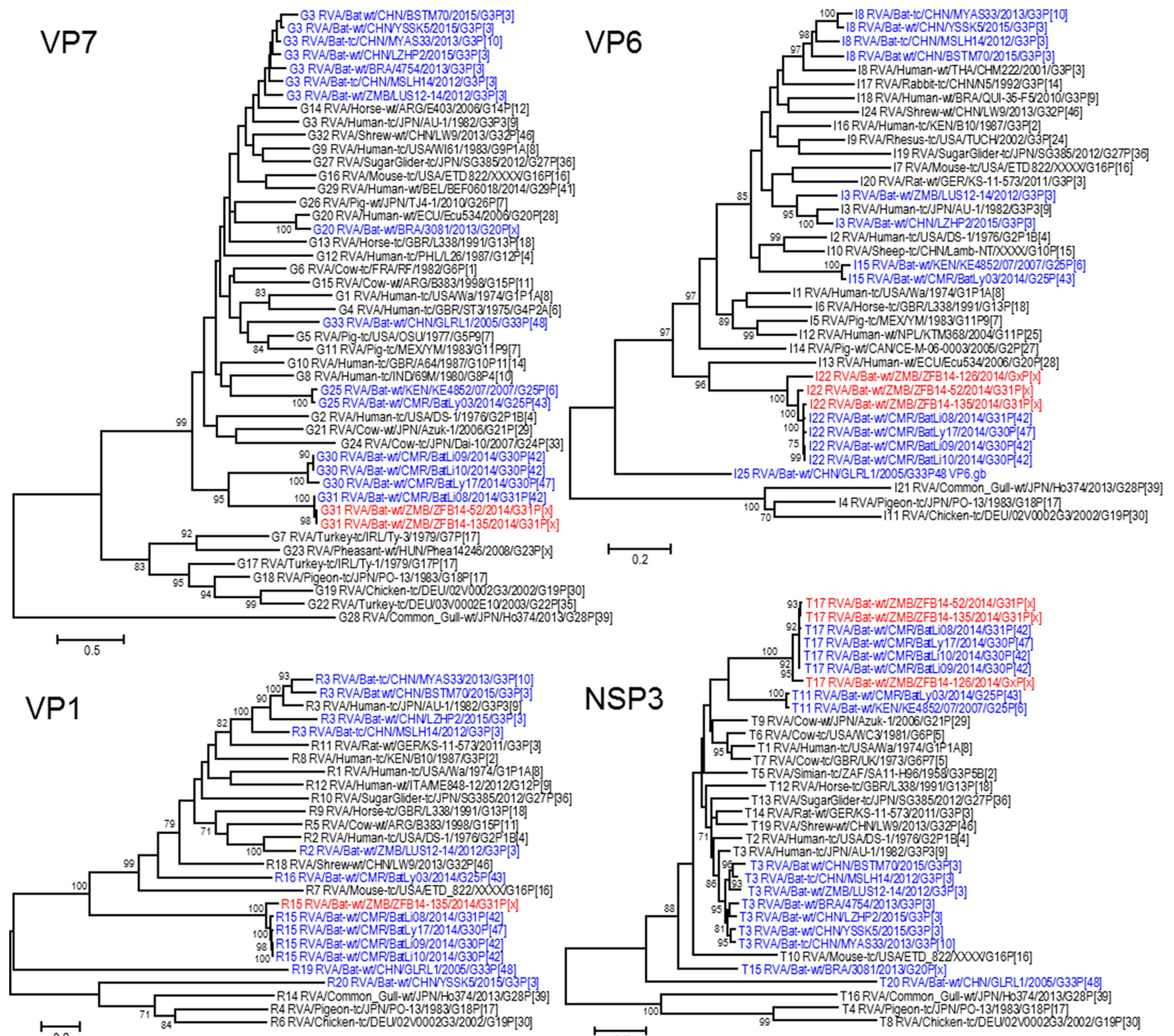

Fig. 1. Phylogenetic analysis based on the nucleotide sequences of the *VP7*, *VP6*, *VP1* and *NSP3* genes.
The group A rotavirus (RVA) strains ZFB14-52, -126 and -135 identified in this study are highlighted in red. Other reference bat RVAs included in the analysis are colored in blue. Genotypes are shown to the left of each taxon. The bootstrap values above 70 after 500 replicates are shown at tree nodes. The scale bars represent the numbers of nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

occurred between these two bat species in the past. However, we could not formally exclude the possibility of mixed infection with different RVA strains in this individual bat.

Unfortunately, we failed to recover all RVA gene segments of these RVA strains and their complete genotype constellations remain to be elucidated. Although several universal primer sets targeting the 5′ and 3′ regions of each genome segment were employed to amplify RVA genomes and determine the genotypes (Fujii et al., 2012; Gentsch et al., 1992; Gouvea et al., 1990; Li et al., 2016), there are significant nucleotide mismatches between these primers and recently described bat RVA genomes (Yinda et al., 2016). A high-throughput sequencing approach may help to identify divergent bat RVA genomes and determine the genotype constellations (He et al., 2017; Yinda et al., 2016).

Previous studies reported that bat RVAs carry genome segments closely related to other mammalian RVAs, including human RVAs (Asano et al., 2016; He et al., 2017; Sasaki et al., 2016). In this study, all detected genome segments were divergent to those of other mammalian RVAs and are tentatively considered to be bat-specific. However, Esona et al. recently identified RVA strain KE4852 from *E. helvum*, which carried *VP4* and *NSP4* genes with shorter genetic distances to other mammalian RVAs providing evidence of interspecies transmission between *E. helvum* and other mammal host species (Esona et al., 2010). Therefore, further studies with increasing numbers of specimens are required to evaluate the public health risk of RVA harbored by Zambian bats and to delineate further the genetic diversity and evolutionary history of these viruses.

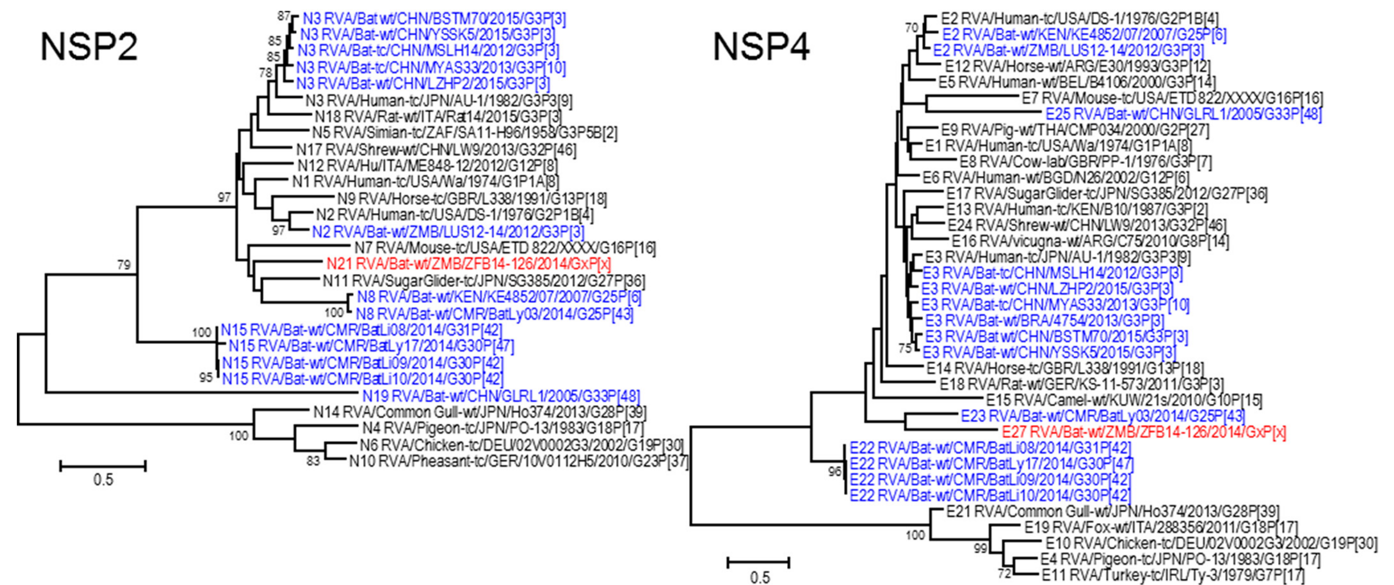

Fig. 2. Phylogenetic analysis based on the nucleotide sequences of the *NSP2* and *NSP4* genes.
The group A rotavirus (RVA) strain ZFB14-126 identified in this study is highlighted in red. Other reference bat RVAs included in the analysis are colored in blue. Genotypes are shown to the left of each taxon. The bootstrap values above 70 after 500 replicates are shown at tree nodes. The scale bars represent the numbers of nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Declaration of interest

The authors declare that they have no conflicts of interest.

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