**Multi-reassortant G3P[3] group A rotavirus in a horseshoe bat in Zambia**

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**Running title**: Interspecies Reassortant Rotavirus in a Bat

**Contents Category**: Animal - Double-strand RNA Viruses

**Word count of the summary**: 108 words

**Word count of the main text**: 1419 words

**Number of tables and figures**: 1 table and 2 figures

**Number of supplementary tables**: 3 supplementary tables

**Footnote**: The GenBank/EMBL/DDBJ accession numbers for the viral sequences reported in this paper are LC158116–LC158126.

**Abstract**

Group A rotavirus is a major cause of diarrhoea in humans, especially in young children. Bats also harbour group A rotaviruses, but the genetic backgrounds of bat rotavirus strains are usually distinct from those of human rotavirus strains. We identified a new strain of group A rotavirus in the intestinal contents of a horseshoe bat in Zambia. Whole genome sequencing revealed that the identified virus, named RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3], possessed the genotype constellation G3-P[3]-I3-R2-C2-M3-A9-N2-T3-E2-H3. Several genome segments of LUS12-14 were highly similar to those of group A rotaviruses identified from humans, cows, and antelopes, indicating interspecies transmission of rotaviruses between bats and other mammals with possible multiple genomic reassortment events.

**Main Text**

Group A rotavirus (RVA) is a major cause of diarrhoeal illness of humans worldwide. The RVA genome comprises 11 segments of double-stranded RNA. Each segment encodes one viral structural protein (VP1, VP2, VP3, VP4, VP6, or VP7) and one nonstructural protein (NSP1, NSP2, NSP3, or NSP4), except for segment 11, which encodes both NSP5 and NSP6. A complete genotype classification system was proposed, defining the genotype constellation of RVAs as follows: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, representing VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 (Matthijnssens *et al.*, 2008). This classification system has facilitated the comparison of various RVA genotypes and increased our knowledge about the genetic diversity of RVA.

Domestic and feral animals as well as humans are susceptible to RVA infection. The three bat-borne RVAs are as follows: RVA/Bat-wt/KEN/KE4852/2007/G25P[6] from a straw-colored fruit bat (*Eidolon helvum*) in Kenya (Esona *et al.*, 2010) and RVA/Bat-tc/CHN/MSLH14/2012/G3P[3] and RVA/Bat-tc/CHN/MYAS33/2013/G3P[10] from a lesser horseshoe bat (*Rhinolophus hipposideros*) and a Stoliczka's trident bat (*Aselliscus storickanus*) in China, respectively (He *et al.*, 2013; Xia *et al.*, 2014). The nucleotide sequences of these bat RVAs are distant from those of other mammalian RVAs and are therefore considered bat-specific, and only the nucleotide sequences of the genes encoding VP4 and NSP4 of RVA/Bat-wt/KEN/KE4852/2007/G25P[6] are highly similar to those of other mammalian RVAs (Esona *et al.*, 2010; He *et al.*, 2013; Xia *et al.*, 2014). Here we identified and characterized a new RVA strain isolated from an insectivorous bat in Zambia.

We captured three horseshoe bats (*Rhinolophus* spp.) and 13 Schreibers' long-fingered bats (*Miniopterus schreibersii*) at Leopard's Hill Cave in Lusaka, Zambia with permission

from the then Zambia Wildlife Authority (Act No. 12 of 1998), now the Department of National Parks and Wildlife, Ministry of Tourism and Arts. None of the bats showed signs of acute infection. They were euthanized by inhalation of diethyl ether, and spleen, lung, kidney, and liver tissues and intestinal contents were collected through dissection. The species were identified according to the nucleotide sequence of the gene encoding mitochondrial cytochrome b, as described previously (Sasaki *et al.*, 2012). For viral metagenomic analysis, intestinal contents from three horseshoe bats were pooled and enriched for viral sequences that were used to generate a library, which was sequenced using the Ion Torrent PGM System (Life Technologies), as described previously (Sasaki *et al.*, 2015). Among 1,163,834 total sequence reads, BLASTN analysis assigned 452 reads to RVA at an e-value cutoff of $10^{-4}$. To screen for the gene encoding RVA VP7 in the 16 captured bats, we used the High Pure Viral RNA Kit (Roche Diagnostics) for extracting nucleic acids from individual intestinal contents, which were then subjected to nested reverse transcription-PCR using the primer sets described by Li *et al*. (Li *et al.*, 2016). RVA nucleotide sequences encoding VP7 were detected in one bushveld horseshoe bat (*Rhinolophus simulator*).

We next determined the nucleotide sequences of 11 genome segments of the detected RVA. The RNA sample was denatured at 98 °C for 2 min in the presence of 1 M betaine and 2.5% DMSO (Darissa *et al.*, 2010), and was then subjected to conventional reverse transcription-PCR, using SuperScript IV Reverse Transcriptase (Life Technologies), Tks Gflex DNA Polymerase (Takara Bio), and specific primers for the sequence reads and universal primers for RVA (Fujii *et al.*, 2012). The 11 genome segments of RVA were sequenced using RNA from the intestinal contents positive for the gene encoding VP7. We then attempted to confirm the 5′- and the 3′-terminal regions of each genome segment using

92 the rapid amplification of cDNA end (RACE) approach with the SMARTer RACE cDNA
93 Amplification Kit (Takara Bio). The 5′-termini of VP4- and VP2-encoding segments and
94 the 3'-termini of VP2- and NSP2-encoding segments were recovered using RACE analysis.
95 Information on all primers used in this study is summarized in Tables S1, S2 and S3
96 (available in the online Supplementary Material). The sequences were deposited in the
97 GenBank/EMBL/DDBJ database under accession numbers LC158116–LC158126.
98 According to the RVA nomenclature proposed by the Rotavirus Classification Working
99 Group (Matthijnssens *et al.*, 2011a), we named the RVA strain as
100 RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3] (LUS12-14). We used RotaC 2.0
101 (http://rotac.regatools.be) to assign LUS12-14 to the
102 G3-P[3]-I3-R2-C2-M3-A9-N2-T3-E2-H3 genotype constellation (Maes *et al.*, 2009). It has
103 been reported that group A rotavirus was detected in some tissues of domestic and
104 experimental animals (Ramig, 2007). To assess the infection of the bat by LUS12-14, we
105 extracted RNA from the spleen, lung, kidney and liver tissues of the bat in which
106 LUS12-14 was detected in the intestinal contents. VP4- and NSP2-endcoding segments of
107 LUS12-14 were exclusively detected in the spleen RNA by RT-PCR using specific primers
108 (Table S2, available in the online Supplementary Material).
109 Table 1 shows the genotype assignment of LUS12-14, the nucleotide positions
110 determined in this study and the nucleotide sequence identities between each segment of
111 LUS12-14 and the strain with the most closely related genome segments. The genome
112 sequences encoding VP7, VP4, VP3, and NSP2 of LUS12-14 shared >97% nucleotide
113 identities with those of RVA/Human-tc/ITA/PA260-97/1997/G3P[3] (Table 1), which was
114 isolated from a child with acute diarrhoea in Italy (De Grazia *et al.*, 2007). We used the
115 maximum likelihood component (500 bootstrap replicates) parameter of MEGA7 software

to deduce the phylogenies of VP7 and VP4 segments according to their nucleotide sequences (Kumar *et al.*, 2016). LUS12-14 VP7 and VP4 clustered with related G3- and P[3]-genotype RVA strains, respectively (Fig. 1). The VP1 genome segment of LUS12-14 exhibited 97.8% nucleotide sequence identity with RVA/Antelope-wt/ZAF/RC-18-08/2008/G6P[14] isolated from a sable antelope with gastroenteritis in South Africa (Matthijnssens *et al.*, 2009). The NSP4 and NSP5 genome segments of LUS12-14 showed 98.0% and 98.6% nucleotide sequence identities with RVA/Cow-wt/ZAF/1604/2007/G8P[1] and RVA/Cow-wt/ZAF/1603/2007/G6P[5], respectively, from the genomes extracted from stool samples of calves with diarrhoea in South Africa (Jere *et al.*, 2012).

The VP6, VP2, NSP1, and NSP3 genome segments of LUS12-14 showed relatively low (<97%) nucleotide sequence identities with RVA sequences deposited in the GenBank/EMBL/DDBJ nucleotide database. Therefore, we deduced the phylogenies of these segments according to their nucleotide sequences. The LUS12-14 VP6 genome segment clustered with I3-genotype RVA strains and was closely related to RVA/Rat-wt/CHN/WC179/2013/G3P[45], the rat RVA strain detected in China (Li *et al.*, 2016) (Fig. 2). The VP2 genome segment of LUS12-14 clustered with the human RVA C2 genotype (Fig. 2). The NSP1 genome segment of LUS12-14 clustered with the A9 genotype of bat RVAs, although it was more closely related to the rabbit strain RVA/Rabbit-tc/CHN/N5/1992/G3P[14] (Guo *et al.*, 2012) (Fig. 2). The NSP3 genome segment of LUS12-14 clustered with the T3-genotype RVA strains and was most closely related to RVA/Human-tc/THA/T152/1998/G12P[9], the human RVA strain in Thailand (Pongsuwanna *et al.*, 2002) (Fig. 2). Moreover, there was no close genetic relationship between LUS12-14 and other bat-derived RVAs.

Complete genome classification studies have revealed the emergence of reassortant RVAs carrying genome segments of RVAs from different mammalian species, suggesting interspecies transmission (Esona *et al.*, 2010; Jere *et al.*, 2012; Li *et al.*, 2016; Matthijnssens *et al.*, 2011b; Matthijnssens *et al.*, 2009). Reassortment contributes to the high genetic diversity of RVA strains. In the present study, we identified and characterized a new RVA strain designated LUS12-14 in the faeces of an insectivorous bat in Zambia. In contrast to known bat-borne RVAs, the LUS12-14 genome comprises segments that are nearly identical or closely related to those of other mammalian RVA strains, suggesting that LUS12-14 represents a multireassortant RVA derived from other mammalian RVA strains that presumably emerged from recent interspecies transmission.

Among the related RVA strains, RVA/Human-tc/ITA/PA260-97/1997/G3P[3] may originate from canine and feline RVA strains (Matthijnssens *et al.*, 2011b), and strains RVA/Cow-wt/ZAF/1604/2007/G8P[1] and RVA/Cow-wt/ZAF/1603/2007/G6P[5] may have been generated through reassortment events between bovine, giraffe, and antelope RVAs (Jere *et al.*, 2012). The identification of LUS12-14 suggests that bats are susceptible to infection by zoonotic RVAs and serve as a host involved in the evolution of RVA through cycles of interspecies transmission accompanied by genome reassortment events.

Although diarrhoea in humans caused by RVA is common in Zambia (Beres *et al.*, 2016; Mpabalwani *et al.*, 2016), little is known about the genotypes of endemic RVA strains of human and other mammals. We detected LUS12-14 in the spleen tissue and faeces of one insectivorous bat, suggesting that LUS12-14 originated from the bat. However, it is unclear whether other bats are infected with LUS12-14 and whether the virus-host relationship detected here is fortuitous. Further studies on LUS12-14 or other zoonotic RVA strains in bats are required to confirm that bats serve as a reservoir of zoonotic RVA strains.

These studies also contribute to our understanding of the evolution of RVA in nature, including that in bats.

**Acknowledgments**

We would like to thank the Department of National Parks and Wildlife of the Ministry of Tourism and Arts (formerly ZAWA) for assistance with sample collection in Zambia. This work was supported in part by the Japan Initiative for Global Research Network of Infectious Diseases (J-GRID) from Japan Agency for Medical Research and Development (AMED), Japan and the Global Institution for Collaborative Research and Education (GI-CoRE) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. This work was supported in part by the Scientific Research on Innovative Areas from MEXT of Japan (16H06429, 16K21723, 16H06431).

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**Table 1. Genotype constellation of rotavirus LUS12-14 and strains with the most closely related segments**

| Gene | Genotype of LUS12-14 | Nucleotide position * | Strains with the most closely related segments | Nucleotide identity (%) |
|---|---|---|---|---|
| VP7 | G3 | 23–1043 | RVA/Human-tc/ITA/PA260-97/1997/G3P[3] | 98.5% |
| VP4 | P[3] | 1–2347 | RVA/Human-tc/ITA/PA260-97/1997/G3P[3] | 97.5% |
| VP6 | I3 | 21–1339 | RVA/Rat-wt/CHN/RA108/2013/G3P[3] | 96.1% |
| VP1 | R2 | 23–3276 | RVA/Antelope-wt/ZAF/RC-18-08/2008/G6P[14] | 97.8% |
| VP2 | C2 | 1–2717 | RVA/Human-wt/GHA/GH018-08/2008/G8P[6] | 96.1% |
| VP3 | M3 | 45–2565 | RVA/Human-tc/ITA/PA260-97/1997/G3P[3] | 97.5% |
| NSP1 | A9 | 26–1531 | RVA/Rabbit-tc/CHN/N5/1992/G3P[14] | 89.5% |
| NSP2 | N2 | 22–1059 | RVA/Human-tc/ITA/PA260-97/1997/G3P[3] | 98.1% |
| NSP3 | T3 | 26–1053 | RVA/Human-tc/THA/T152/1998/G12P[9] | 91.7% |
| NSP4 | E2 | 29–720 | RVA/Cow-wt/ZAF/1604/2007/G8P[1] | 98.0% |
| NSP5 | H3 | 21–642 | RVA/Cow-wt/ZAF/1603/2007/G6P[5] | 98.6% |

***The nucleotide positions correspond to those of the Wa strain (RVA/Human-tc/USA/Wa/1974/G1P[8], JX406747–JX406757).**

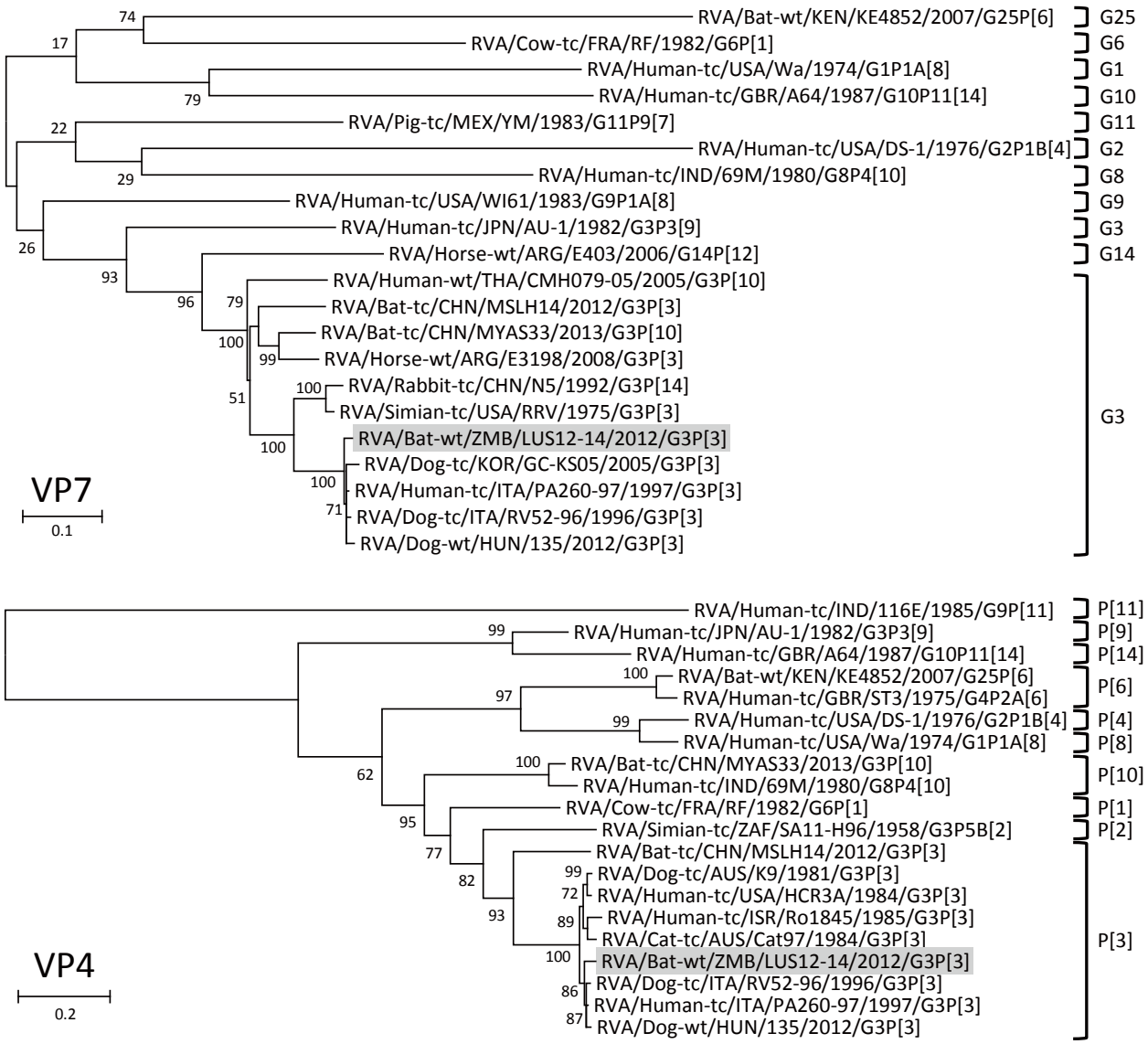
## Figure Legends

**Fig. 1. Phylogenetic analyses of the genes encoding VP7 and VP4**

The rotavirus strain LUS12-14 identified in this study, its related strains, and the representative reference strains were included in the analysis. LUS12-14 is shaded gray. Genotypes are shown to the right of the trees. The bootstrap values obtained after 500 replicates are indicated at major tree roots. The scale bars represent the numbers of nucleotide substitutions per site.

**Fig. 2. Phylogenetic analyses of the genes encoding VP6, VP2, NSP1 and NSP3**

The rotavirus strain LUS12-14 identified in this study, its related strains, and the representative reference strains were included in the analysis. The analysis is described in the legend for Fig. 1.


RVA/Bat-wt/KEN/KE4852/2007/G25P[6]
RVA/Cow-tc/FRA/RF/1982/G6P[1]
RVA/Human-tc/USA/Wa/1974/G1P1A[8]
RVA/Human-tc/GBR/A64/1987/G10P11[14]
RVA/Pig-tc/MEX/YM/1983/G11P9[7]
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]
RVA/Human-tc/IND/69M/1980/G8P4[10]
RVA/Human-tc/USA/WI61/1983/G9P1A[8]
RVA/Human-tc/JPN/AU-1/1982/G3P3[9]
RVA/Horse-wt/ARG/E403/2006/G14P[12]
RVA/Human-wt/THA/CMH079-05/2005/G3P[10]
RVA/Bat-tc/CHN/MSLH14/2012/G3P[3]
RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]
RVA/Horse-wt/ARG/E3198/2008/G3P[3]
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RVA/Simian-tc/USA/RRV/1975/G3P[3]
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RVA/Dog-tc/KOR/GC-KS05/2005/G3P[3]
RVA/Human-tc/ITA/PA260-97/1997/G3P[3]
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RVA/Dog-wt/HUN/135/2012/G3P[3]
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G6
G1
G10
G11
G2
G8
G9
G3
G14
G3
VP7
0.1
RVA/Human-tc/IND/116E/1985/G9P[11]
RVA/Human-tc/JPN/AU-1/1982/G3P3[9]
RVA/Human-tc/GBR/A64/1987/G10P11[14]
RVA/Bat-wt/KEN/KE4852/2007/G25P[6]
RVA/Human-tc/GBR/ST3/1975/G4P2A[6]
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]
RVA/Human-tc/USA/Wa/1974/G1P1A[8]
RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]
RVA/Human-tc/IND/69M/1980/G8P4[10]
RVA/Cow-tc/FRA/RF/1982/G6P[1]
RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2]
RVA/Bat-tc/CHN/MSLH14/2012/G3P[3]
RVA/Dog-tc/AUS/K9/1981/G3P[3]
RVA/Human-tc/USA/HCR3A/1984/G3P[3]
RVA/Human-tc/ISR/Ro1845/1985/G3P[3]
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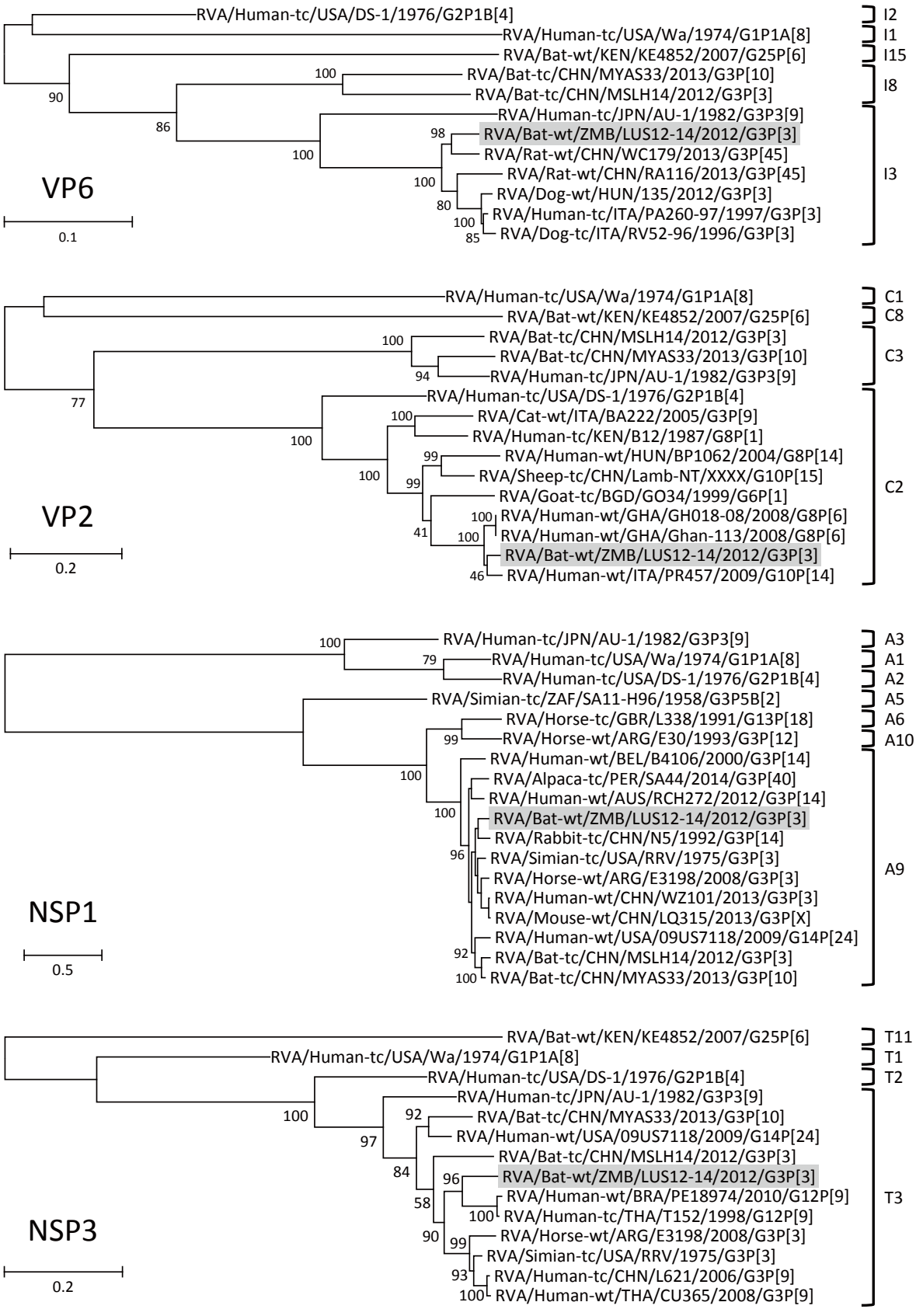
P[1]
P[2]
P[3]
VP4
0.2

Fig. 1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RVA/Bat-tc/CHN/MSLH14/2012/G3P[3]
RVA/Human-tc/JPN/AU-1/1982/G3P3[9]
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]
RVA/Rat-wt/CHN/WC179/2013/G3P[45]
RVA/Rat-wt/CHN/RA116/2013/G3P[45]
RVA/Dog-wt/HUN/135/2012/G3P[3]
RVA/Human-tc/ITA/PA260-97/1997/G3P[3]
RVA/Dog-tc/ITA/RV52-96/1996/G3P[3]
I2
I1
I15
I8
I3
VP6
0.1
RVA/Human-tc/USA/Wa/1974/G1P1A[8]
RVA/Bat-wt/KEN/KE4852/2007/G25P[6]
RVA/Bat-tc/CHN/MSLH14/2012/G3P[3]
RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]
RVA/Human-tc/JPN/AU-1/1982/G3P3[9]
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]
RVA/Cat-wt/ITA/BA222/2005/G3P[9]
RVA/Human-tc/KEN/B12/1987/G8P[1]
RVA/Human-wt/HUN/BP1062/2004/G8P[14]
RVA/Sheep-tc/CHN/Lamb-NT/XXXX/G10P[15]
RVA/Goat-tc/BGD/GO34/1999/G6P[1]
RVA/Human-wt/GHA/GH018-08/2008/G8P[6]
RVA/Human-wt/GHA/Ghan-113/2008/G8P[6]
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]
RVA/Human-wt/ITA/PR457/2009/G10P[14]
C1
C8
C3
C2
VP2
0.2
RVA/Human-tc/JPN/AU-1/1982/G3P3[9]
RVA/Human-tc/USA/Wa/1974/G1P1A[8]
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]
RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2]
RVA/Horse-tc/GBR/L338/1991/G13P[18]
RVA/Horse-wt/ARG/E30/1993/G3P[12]
RVA/Human-wt/BEL/B4106/2000/G3P[14]
RVA/Alpaca-tc/PER/SA44/2014/G3P[40]
RVA/Human-wt/AUS/RCH272/2012/G3P[14]
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]
RVA/Rabbit-tc/CHN/N5/1992/G3P[14]
RVA/Simian-tc/USA/RRV/1975/G3P[3]
RVA/Horse-wt/ARG/E3198/2008/G3P[3]
RVA/Human-wt/CHN/WZ101/2013/G3P[3]
RVA/Mouse-wt/CHN/LQ315/2013/G3P[X]
RVA/Human-wt/USA/09US7118/2009/G14P[24]
RVA/Bat-tc/CHN/MSLH14/2012/G3P[3]
RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]
A3
A1
A2
A5
A6
A10
A9
NSP1
0.5
RVA/Bat-wt/KEN/KE4852/2007/G25P[6]
RVA/Human-tc/USA/Wa/1974/G1P1A[8]
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]
RVA/Human-tc/JPN/AU-1/1982/G3P3[9]
RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]
RVA/Human-wt/USA/09US7118/2009/G14P[24]
RVA/Bat-tc/CHN/MSLH14/2012/G3P[3]
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]
RVA/Human-wt/BRA/PE18974/2010/G12P[9]
RVA/Human-tc/THA/T152/1998/G12P[9]
RVA/Horse-wt/ARG/E3198/2008/G3P[3]
RVA/Simian-tc/USA/RRV/1975/G3P[3]
RVA/Human-tc/CHN/L621/2006/G3P[9]
RVA/Human-wt/THA/CU365/2008/G3P[9]
T11
T1
T2
T3
NSP3
0.2

Fig. 2