At least seven distinct rotavirus genotype constellations in bats with evidence of reassortment and zoonotic transmissions

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ABSTRACT

Bats host many viruses pathogenic to humans, and increasing evidence suggests that Rotavirus A (RVA) also belongs to this list. Rotaviruses cause diarrheal disease in many mammals and birds, and their segmented genomes allow them to reassort and increase their genetic diversity. Eighteen out of 2,142 bat faecal samples (0.8%) collected from Europe, South America and Africa were PCR-positive for RVA and 11 of those were fully characterized using viral metagenomics. Upon contrasting their genomes with publicly available data, at least 7 distinct bat RVA genotype constellations were identified, including evidence of reassortments among them. Some of these constellations are spread across the world, whereas others appear to be geographically restricted. Our analyses also provide evidence for multiple zoonotic transfer events involving bat RVAs. A Bulgarian bat RVAs possessed a genotype constellation previously identified in Chinese bats, and identical to a rare Argentinean horse RVA. A Costa Rican bat RVA possessed 3 previously undescribed gene segments and clustered closely with a human strain. Although SA11 is one of the most widely used reference strains for RVA research and forms the backbone of a reverse genetics system, its origin remained enigmatic. Remarkably, the majority of the gene segments of SA11 were closely related to Gabonese bat RVAs, suggesting a potential bat origin. Overall, our findings suggest an underexplored genetic diversity of RVAs in bats which is likely the tip of the iceberg. Increasing contact between humans and bat wildlife will further increase the zoonosis risk, which warrants closer attention to these viruses.

Importance

The increased research on bat coronaviruses after SARS-CoV and MERS-CoV, allowed the very rapid identification of SARS-CoV-2. This is an excellent example of the importance of knowing viruses harboured by wildlife in general and bats in particular, for global preparedness against emerging viral pathogens. The current effort to characterise bat rotavirus strains from 3 continents provided evidence that several atypical rotaviruses in humans and animals might have a bat origin, implying that zoonoses of bat rotaviruses occur more frequently than currently realized.

Keywords: Viral metagenomics, bat rotavirus, rotavirus genetic diversity, SA11, zoonosis

Author Contributions

C.D, J.F.D, J.M and M.V.R designed the research; V.M.C., H.U.E., A.N.L., A.R., G.D.M., T.B., F.G.R., A.SH., S.Y., A.S, S.O., Y.A.S., P.V., M.B. and E.M.L. were involved in sample collection; V.M.C., H.U.E., A.N.L. and C.S. performed the research; C.S., D.J., L.B., W.D., H.U.E., V.M.C. and K.C.Y. contributed in data analysis; J.F.D., C.D., C.S. and J.M. drafted the paper; final version was approved by all co-authors.

INTRODUCTION

Rotaviruses are the leading cause of diarrheal disease in the young of mammals and birds. In humans, rotaviruses are responsible for 122,000-216,000 deaths in under 5-year old infants on a yearly basis, mainly in developing countries (1). The *Rotavirus* genus belongs to the family *Reoviridae* and contains 9 species designated as A-I (RVA-RVI). The rotavirus genome consists of 11 dsRNA segments encoding 6 structural viral proteins (VP1-6) and 6 non-structural proteins (NSP1-6) (2).

The RVA outer capsid antigens, VP4 and VP7 are used for a dual classification system defining P-genotype (VP4 is Protease sensitive) and G-genotype (VP7 is Glycosylated), respectively (2). However, as gene reassortment is a common phenomenon for viruses with a segmented genome after co-infection, a more comprehensive classification approach became necessary to better account for the genome evolution and genetic diversity of RVAs. In 2008, a nucleotide sequence-based, complete genome classification system was developed for RVA, define genotypes for each of the 11 gene segment. These genotypes allowed extending the dual classification to full 'genotype constellations' classification (3, 4). The gene assignments are reported as Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, where 'x' denotes the particular genotype. The Rotavirus Classification Working Group (RCWG) was formed in order to assign new genotypes to rotavirus genes which could not be assigned to an established genotype (5). A web-based RotaC tool that uses the pre-set nucleotide cut-off values was also developed for automated genotype assignment (6).

Accumulating whole genome sequencing data demonstrate that there are typical genotype constellations present in most animal species. Two of them, Wa-like and DS-1-like, are responsible for most of the human disease and designated as I1-R1-C1-M1-A1-

113 N1-T1-E1-H1 and I2-R2-C2-M2-A2-N2-T2-E2-H2, respectively, for the non-G/P
114 genotypes (3). Furthermore, various animal species are known to have specific genotype
115 constellations such as I2-R2-C2-M2-A3/A11/A13-N2-T6-E2-H3 for cattle and other even-
116 toed ungulates (7), I1/I5-R1-M1-A1/A8-N1-T1/T7-E1-H1 for swine (3, 8), I2/I6-R2-C2-M3-
117 A10-N2-T3-E2/E12-H7 for horses (9), and I3-R3-C3-M3-A3/A9-N2-T3-E3-H3/H6 for cats
118 and dogs (10). Partially shared genotype patterns between established genotype
119 constellations, such as Wa-like human RVA strains and porcine RVAs, as well as DS-1-
120 like human RVA strains and bovine RVAs, suggest a common origin and important
121 zoonotic transfer events in the past (3).
122 Bats belong to the Chiroptera order, which is the second largest order of mammals (11).
123 They harbour a high diversity of viruses, among them are also zoonotic viruses such as
124 lyssavirus, Hendra and Nipah viruses, filovirus and several coronaviruses (12–18). These
125 viruses can be transmitted to humans via saliva, infected tissues, faeces and direct
126 contact (19). Given their great population densities, migration ability and proximity to
127 human habitats; bats are often screened for emerging and re-emerging viral pathogens
128 (20, 21). Such screenings have resulted in the sporadic identification of rotavirus strains
129 in bats in the last decade. Even though there are reports of RVH in South Korean bats in
130 2016 (22) and Cameroonian bats in 2018 (23), and a novel rotavirus species (tentatively
131 named RVJ) was identified from Schreiber's bats in Serbia in 2014 (24); RVA is the most
132 commonly detected species and there are currently more than 20 bat RVA strains
133 identified in literature. In 2010, Esona and colleagues reported the first partially sequenced
134 RVA strain (KE4852) in a Kenyan *Eidolon helvum* (straw-coloured fruit bat), and the
135 majority of retrieved gene segments were only distantly related to known mammalian RVA
136 strains, representing novel genotypes (25). During the subsequent decade, sporadic and

scattered reports have been published about RVA strains in bats collected from serum, gut and faecal samples in insectivorous and fruit bats. Several reports of bat RVA strains came from Chinese studies (26–29), but bat RVAs were also detected and (partially) characterized from France (30), Brazil (31), Zambia (32, 33), Cameroon (34), Kenya (35) and Saudi-Arabia (36). These studies investigate samples from a variety of different bat species such as *Rhinolophus hipposideros* (lesser horseshoe bat) (26), *Aselliscus stoliczkanus* (Stoliczka's trident bat) (27), *Myotis mystacinus* (whiskered bat) (30), *Molossus molossus* (velvety free-tailed bat), *Glossophaga sorcina* (Pallas's long-tongued bat) (31), *Rhinolophus simulator* (Bushveld horseshoe bat) (32), *Hipposideros pomona* (Pomona roundleaf bat), *Taphozous melanopogon* (black-bearded tomb bat), *Scotophilus kuhlii* (lesser Asiatic yellow bat), *Rousettus leschenaultii* (Fulvous fruit bat) (28), *Taphozous mauritianus* (Mauritian tomb bat) (35), *Rousettus aegyptiacus* (Egyptian fruit bat) (33, 35), *Taphozous perforatus* (Egyptian tomb bat), *Rhinopoma hardwickii* (lesser mouse-tailed bat) (36) and *Eidolon helvum* (25, 33, 34, 36). From some of these novel bat RVA strains a few gene segments were sequenced, whereas other strains were sequenced completely, often resulting in one or multiple novel genotypes (25, 28, 31, 33, 34).

Even though RVAs are generally considered to have a rather restricted host range, a number of unusual strains have been described in literature, suggestive of interspecies transmissions involving bat RVA strains. One example is the E3198 strain that was isolated from a diarrheic foal in Argentina in 2008 (37). Although its genotype constellation was distantly related to feline/canine-like RVA strains at that time, 2 more recent publications showed a closer relationship with Chinese bat RVA strains in several gene segments (26, 27). A second example was the unusual human G3P[3] RVA strain 12638,

isolated from a 4 year-old child with severe gastroenteric symptoms in Japan in 2014. Three out of its 11 gene segments were closely related to a South African bat RVA strain, suggesting a reassortment involving a bat RVA strain (38). A third example is two unique G20 human RVA strains, Ecu534 from Ecuador (39) and 2014735512 from Suriname (40). The recent identification of the G20 genotype in a Brazilian bat RVA strain (3081) also suggests a potential bat reservoir for these human strains (31).

All in all, slowly emerging data on bat RVA strains start to show that some unusual previously identified human and animal RVA strains might actually have been derived from bats. Therefore, the constant surveillance of novel and reassortant RVA bat strains from all over the world has to continue in order to better understand the genetic diversity of bat RVA strains, as well as to maintain both public and animal health. Here we report identification of 11 bat RVA strains from Bulgaria, Gabon, Ghana and Costa Rica, providing evidence of multiple reassortment and host switching events from bats to bats and to other mammals.

RESULTS

Bat rotavirus screening from Europe, Africa and South America samples

As part of several studies screening bat picornaviruses, astroviruses, coronaviruses and paramyxoviruses, bat faecal samples from Bulgaria, Romania, Germany, Gabon, Ghana and Costa Rica were previously collected (41–45). In the current study, these samples were screened for RVA, using a nested RT-PCR targeting a short piece of the highly conserved polymerase gene (VP1). This screening yielded 18 positives out of the 2,142 screened samples (0.8%) (Table S2). RVA positive samples were collected from five bat

185 families Pteropodidae, Rhinolophidae, Hipposideridae, Phyllostomidae and
186 Vespertilionidae, and they originated from three continents and all sampling sites except
187 Romania.
188
189 **Eleven near complete RVA genomes identified from 4 bat families**
190 From 16 of the positive samples, a sufficient amount of sample was available for complete
191 viral genome sequencing using the NetoVIR protocol (Table S3). 118,9 million paired-end
192 reads (2x150 base pairs) and an average of 7 million paired-end reads/sample were
193 generated by Illumina sequencing (Table 1). Four samples from Gabon and 1 sample from
194 Germany did not yield any RVA contigs longer than 500 base pairs and were therefore
195 not investigated further. From 11 samples near complete RVA genomes could be
196 retrieved. These RVA samples belonged to 5 out of the 46 tested species (10.8%), from
197 4 out of the 10 (40%) tested families, as shown in the bat phylogenetic tree (Table S2,
198 Figure S1). The percentage of reads mapping to RVA in each sample ranged from 0-90%
199 (Table 1).
200
201 **Distinct and reassorted RVA genotype constellations with 4 novel genotypes**
202 The genotype constellations of the 11 bat RVA strains are shown in Table 2. The genotype
203 assignments, including novel NSP2 (N23) and NSP4 (E28) genotypes for some of the
204 Gabonese strains and NSP1 (A32) and NSP3 (T23) genotypes for the strains from Costa
205 Rica were made according to the guidelines determined by the RCWG (46). Although the
206 NSP5 gene segment of the Costa Rican strain KCR10-93 most likely also represents a
207 novel genotype, we were not able to retrieve the complete ORF (despite several attempts
208 using RT-PCR and Sanger sequencing), which is required for the assignment of a novel

genotype (47). Particular genotype constellations were identified in different geographic locations (Table 2). Gabonese strains were similar to each other, with certain genotypes shared with the Bulgarian strains (G3, P[3], C3, M3, N3, T3 and E3). However, they do not cluster closely together (*vide supra*), indicating non-recent reassortment events. The South American strain KCR10-93 also possessed a unique genotype constellation, except for the VP4 genotype P[47], which was shared with the Ghanaian strain. Interestingly, these 2 VP4 genes were very closely related (*vide supra*), suggesting a recent reassortment event. GKS-912, GKS-926 and GKS-934 appeared to have a co-infection, as multiple genotypes were identified in these samples for VP2, VP3, VP4, NSP2, NSP3 and NSP4. For GKS-934, 2 near complete VP7 gene segments were identified, both belonging to the G3 genotype, yet having a substantial nucleotide level dissimilarity (19%, *vide infra*). This was also the case for K212 possessing 2 distinct M14 genotypes with 12% nucleotide sequence distance.

At least 7 seven distinct bat RVA constellations distributed worldwide

Even though most animal species, including humans, have a limited number of typical RVA genotype constellations, the RVAs harboured by bats show a great genetic diversity. Combining our data with previously published bat RVA genomes showed that there are at least 7 distinct bat RVA genotype constellations circulating in the bat population (Table 3), ranging from completely unique to partially overlapping with each other. The Bulgarian BB89-15 and BR89-60 strains had a genotype constellation identical or very similar to MSLH14-like RVA strains from China and a partially sequenced strain from Brazil (“orange” genotype constellation in Table 3). Even though at least 3 of the samples from Gabon possessed more than one RVA strain, they possessed at least 3 distinct but related

genotype constellations ("purple" genotype constellation in Table 3), not previously identified in bats. It should be noted that there was some genotype overlap for gene segments VP2-VP4, VP7, NSP2-NSP4 between the orange and purple genotype constellations, with varying level of phylogenetic relatedness (*vide infra*). Ghanaian strain K212 possessed a genotype constellation ("green" genotype constellation in Table 3) identical or very similar to several previously identified Cameroonian bat RVA strains (34), as well as some partially sequenced bat RVA strains from Zambia (33). Costa Rican RVA strain KCR10-93 had a distinct genotype constellation ("brown" genotype constellation in Table 3), including at least 2 previously undescribed genotypes, and shared the G20 genotype with the Brazilian bat strain 3081. Of interest was the P[47] genotype, which was shared with 2 African strains from the green genotype constellation. The "yellow" genotype constellation in Table 3 was composed of 2 strains with an identical genotype constellation from Cameroon (BatLy03) and Saudi Arabia (KSA402), as well as a partially sequenced strain from Kenya (KE4852). Two genotype constellations (indicated in "blue" and "dark grey" in Table 3) were only represented by a single bat strain from Kenya (BATp39) and China (GLRL1), respectively (Table 3). Of note, the former shared the VP6 genotype (I16) with the purple genotype constellation, and for the latter strain, the NSP1 sequence remained undetermined.

Wide geographic dispersal of certain bat RVA genotype constellations

The global distribution of the RVA genotype constellations revealed several patterns regarding RVA circulation in bats, as shown in Figure 1. Bat RVAs belonging to the brown, purple, blue and dark grey genotype constellations have so far only been identified in Costa Rica (and perhaps Brazil), Gabon, Kenya and China, respectively. On the other

hand, the green and yellow genotype constellations were confirmed to be further dispersed, from Cameroon to Saudi Arabia (G25P[43]), and from Ghana and Cameroon to Zambia, as was previously suggested by Sasaki *et al.* (48). However, highly similar RVA strains belonging to the orange MSLH14-like genotype constellations span at least 3 different continents and subcontinents, e.g. Asia, Europe and possibly South America. Furthermore, it was also shown that RVA strains with distinct genotype constellations could co-circulate in the same region, as is the case in Cameroon (green, yellow and purple genotype constellations) and China (orange and dark grey genotype constellations) (Figure 1).

Interspecies transmission in bats and potential host range restriction of bat RVAs

The orange genotype constellation was present in various bat families. The Bulgarian RVA strains were isolated from rhinolophid bats, whereas the Chinese MSLH14-like strains were found in bats from the Rhinolophidae, Hipposideridae and Emballonuridae families (Table S4a).

In addition to RVA genotype constellations potentially being able to infect multiple bat families, individual bat families could also harbour more than one genotype constellation, as is shown in Table S3b. Bat RVA strains with the green (Ghana and Cameroon) and yellow (Cameroon and Saudi Arabia) genotype constellations have both been found in straw-coloured fruit bats.

Reassortments among bat RVA strains

Even though the genotype constellations are somewhat conserved, there are ample examples for the occurrence of reassortments. In the orange genotype constellation, there

are some unusual genotypes such as P[10] for VP4, R20 for VP1 and A29 for NSP1 (Table S4a and S4c) which are most likely the results of reassortment events with currently unknown RVA strains (27, 28). Reassortment also takes place between different bat RVA genotype constellations, albeit to a limited extension. For example, GKS-897 is the only strain from the purple genotype constellation with the I8 VP6 genotype, which is shared with several strains from the orange genotype constellation (MSLH-14, BSTM70, MYAS33 and YSSK5), suggesting a reassortment event. A second example is the I16 VP6 genotype, which is shared between BATp39 from Kenya (the only member of the blue genotype constellation), and most strains of the purple genotype constellation. A third example is the shared P[47] VP4 genotype between K212 and BatLy17 (green genotype constellation) and KCR10-93 (brown genotype constellation) (Table 2). Interestingly, these last 3 strains were 97-100% identical to each other on the nucleotide level for VP4, suggesting a recent reassortment event. Finally, there are also a few bat RVA strains with unusual genotype constellations, which do not clearly fall into the 7 described genotype constellations. RVA strains LUS12-14 and YSSK5, from Zambia and China respectively, possess several genotypes typical for the orange genotype constellation, in addition to several other genotypes of unknown origin (Table S4c). Finally, strain 322/Kwale from Kenya possesses both genotypes typical to the orange and purple genotypes, in addition to some atypical bat RVA genotypes.

Evidence of interspecies transmissions of bat RVA to humans, horses and potentially non-human primates

We further investigated whether unusual RVA strains detected in other mammals (including humans) might be a result of an interspecies transmission from bat strains identified in the current and other studies (Table 3).

RVA/Horse-wt/ARG/E3198/2008/G3P[3]

Although it had already been suggested that the unusual horse RVA strain E3198 was of potential bat origin, our data provided further and more compelling evidence for this, as strain E3198 showed an identical genotype constellation with the two Bulgarian bat RVA strains. They displayed a close phylogenetic clustering for all 11 gene segments (Figure S2, Figures 2-4) and shared very high nucleotide similarities (87-97%). Our dataset was not optimally suited for molecular clock analysis, but extrapolation of substitution rates of about 0.6×10^{-3} – 1.5×10^{-3} substitutions/site/year (s/s/y) inferred from analyses of human RVA (49, 50) suggests that the zoonotic transfer could have taken place within the last few decades.

RVA/Simian-tc/ZAF/SA11-N2/1958/G3P[2]

The unusual simian RVA SA11 has been a reference strain for many rotavirus studies for decades. Two unusual human RVA strains, ZTR-5 and B10 have 10 and 8 genotypes in common with SA11, respectively (Table 3). The former strain has only been deposited in GenBank (without any further discussion), whereas the latter was believed to be the result of zoonotic transmission from monkeys to humans (51). To our surprise, these 3 strains were clearly related to the purple bat genotype constellation identified in our study, sharing up to 9 (B10) and 7 (SA11 and ZTR-5) genotypes (Table 3). For the segments sharing the

same genotype the time of the most recent common ancestor or zoonotic transfer was estimated to be a few decades to centuries ago.

Also, according to the pairwise nucleotide identities (Figure S2) and the phylogenetic analyses of the bat RVA strains from Gabon and Kenya, they were closely related with B10 for the VP1, VP6, NSP4 gene segments, and with all 3 strains (B10, SA11 and ZTR-5) for VP2-4, NSP1, NSP3 and NSP5 (Figures 2-4).

Not only for SA11, but also for 2 other simian RVA strains, RRV and TUCH, some close relationships with bat RVA strains were noted. The VP1, VP3, VP4, VP6, VP7, NSP1-5 gene segments of RRV clustered closely with one or multiple bat and bat-related RVA strains (Figures 2-4). For TUCH, the VP1, NSP1, NSP5 gene segments also clustered close to bat RVA strains (Figures 2-4). These findings suggest that these simian RVA strains might also have a common ancestor with bat RVA strains.

Human-Bat RVA interspecies transmission

Two unusual G20 human RVA strains (Ecu534 and 2014735512) were isolated in 2006 and 2013, respectively in Central America. Costa Rican bat RVA strain KCR10-93 shared multiple genotypes (Table 3), and medium-high nucleotide similarities (Figure S2) with 2014735512. The most recent common ancestor between the Costa Rican bat and the human RVA strains was estimated no longer than a few centuries ago. In addition, the available gene segments cluster relatively closely together (Figures 2-4) phylogenetically. It should also be noted that the T23 NSP3 genotype is phylogenetically closely related to the T15 genotype found in the human strains, and the currently unclassified partial NSP5 sequence also has the H15 genotype, as its closest relative.

Furthermore, 2 unusual human G3P[9] RVA strains (L621 and E2451), that were detected in China in 2006 and 2011, respectively, had been speculated to be of animal/bat origin. Our data further adds support to this hypothesis, as either both or one of them were closely related to the identified Bulgarian strains for VP3, VP6 and NSP4 (Figures 2-4). Finally, the unusual human RVA strain RVA/Human-wt/US/09US7118/2009/G3P[24] was also found to cluster together with one or multiple bat RVA strains for its VP2, VP7, NSP2, NSP3, NSP4, NSP5 gene segments. Furthermore, its VP3, VP4, VP6 and NSP3 gene segments clustered closely with the TUCH strain, also hinting at a potential bat origin of this unusual human strain (*vide supra*).

DISCUSSION

Bats are known hosts of various human pathogens, including viruses such as rabies virus, henipaviruses, Marburg virus, SARS and MERS CoVs (12–18). In addition, there have been sporadic reports on several other RNA viruses in bats such as paramyxoviruses, picornaviruses, orthoreoviruses and astroviruses (52–55). Bat rotaviruses have also been sporadically reported during the last decade. A novel species, tentatively named rotavirus J, was isolated from *M. schreibersii* in 2014, followed by rotavirus H, in 2016 and 2018 (22–24). Nonetheless, it was rotavirus A (RVA) that has been the most frequently reported rotavirus species in bats. This is not very surprising given the fact that RVA has been detected in a wide range of mammals and birds (56–58). Furthermore, there are plenty of examples of this enteric pathogen being capable of interspecies transmission in literature, sometimes in combination with reassortment, between various mammalian species including humans (59). In some occasions, such animal-derived gene segments (e.g. VP7

373 genotypes G8 from cattle, G9 and presumably G12 from pigs) or complete genotype
374 constellations (AU-1 like genotype constellations from cats) have become established in
375 the human population. This established circulation either happened in a limited
376 geographical region (AU-1 like or G8) or worldwide; such as epidemiologically important
377 human pathogenic G9 and G12 RVAs (60, 61). It is important to note that even when there
378 is no clinical presentation in the animal of which the enteric pathogen has been identified,
379 RV shedding can still contribute to zoonoses (59). Thus, in order to further investigate the
380 potential of bat RVA strains to spill over between bat species or towards other mammalian
381 species, we investigated RVA strains from over 2,000 bats, spanning 5 countries in 3
382 continents. We obtained 11 near complete RVA genomes with a viral metagenomics
383 approach and the identified genomes were compared and contrasted to known genome
384 sequences of bat and other host species. We aimed to expand the knowledge on typical
385 bat genotype constellations as well as their geographical spread and zoonotic potential.
386
387 **Driving and restricting forces of bat RVA genetic diversity**
388 Bat RVA strains with at least 7 largely distinct genotype constellations have been identified
389 up to date (Table 3). This does not come as a surprise given the breadth of this order of
390 animals and many more bat RVA strains and novel genotype constellations will be
391 discovered in years to come. In the current study, some of these 7 genotype constellations
392 have only been found in a single bat (and hence a single location), whereas some
393 genotype constellations have been found in bats living thousands of kilometres apart
394 (green and yellow genotype constellations), or even on multiple continents (orange
395 genotype constellation). Bats have very specialized wing and tail structures for better
396 manoeuvring and higher flexibility during flight (62). With powered flight, migratory bats

can travel long distances between summer and winter roosts, for foraging and searching for a mate (63). Among long-distance migratory bats, *E.helvum* can cover a range of 270 to 2,500 km (64), vespertilionid 'tree bats' and the subtropical/tropical molossid bats can fly over 1,000 km (65, 66). Global distribution and intercontinental bat virus transfers are also typical to other bat viruses (43). In addition to migration across vast distances, the fact that some distinct genotype constellations seem to have overlapping geographical ranges (such as in China and West Africa in Figure 1) suggest some type of fitness advantage for these particular genotype constellations. However, there is also ample evidence of gene reassortment events among established genotype constellations (e.g. P[47] in green and brown genotype constellation; or I16 in purple and blue genotype constellation), or with RVA strains of currently unknown origin (e.g. A29, A15, E27).

Apart from geographical location, host physiology and behaviour can also be factors affecting the viral epidemiology and risk for host switches. As demonstrated by the wide dispersal of the orange constellation, RVAs belonging to certain bat families might undergo multiple host switching events. Although dietary habits may not directly determine the type of pathogens bats carry, it can bring different species close together, providing plenty of opportunity for interspecies transmissions and subsequent reassortment events to occur (19). Pteropodid bats harbour completely unique genotype constellations (green and yellow), suggesting that the associated RVA strains had high epidemiologic fitness in these populations. This further indicates that the Pteropodidae, which includes the straw-coloured fruit bats, has been a substantial virus reservoir for a long time already, as also shown for Marburg virus, Hendra and Nipah viruses (13–15).

It is clear that more bats should be sampled in order to have a comprehensive understanding of the driving and restricting forces, or the lack thereof. The detection of

P[47] reassortment between Ghanaian and Costa Rican bat RVAs, which are located more than 9,000 km's apart, cannot only be explained by the flight ability of bats, but rather the lack of sampling between these 2 locations. We hypothesize that with the increasing bat RVA sequencing efforts, the geographical and host range of most genotype constellations (such as the blue, grey, yellow and brown) will be significantly expanded.

Interspecies transmission of bat RVAs to mammalian hosts

Bat RVA transmission to a horse

In 2013, Miño and colleagues reported an unusual Argentinian equine G3P[3] RVA strain E3198. Based on the genotype constellation, it was speculated to have a common ancestor with both feline/canine RVA strains, as well as the unusual rhesus RVA strain RRV. However, the nucleotide identities were below the 90% for most of the genome segments, suggesting that the original host may not be identified yet (37). When more bat RVA genomes became available in subsequent years, Xia and colleagues, and later also Biao He and colleagues, suggested that E3198 might be of bat origin, based on the genotype constellations and nucleotide similarities (27, 28). However, the very close genetic relationship between E3198 and the Bulgarian strains presented in this paper across all 11 gene segments (Figure S4) seems to provide final proof for the bat origin of this unusual equine RVA strain.

Bat RVA transmission to simians and humans

RVA strain SA11 was isolated from an overtly healthy vervet monkey in 1958 and has subsequently been used extensively as a laboratory strain in RVA growth, virulence, genome replication and in recent years also a reverse genetics research (67–69).

445 However, its origin remained obscure, as related strains were never identified in vervet
446 monkeys or other non-human primates ever after. In 2011, Ghosh and colleagues
447 identified an unusual RVA strain B10 from a child in Kenya, which shared 8 out of 11
448 genotypes with SA11. They speculated about a simian or other animal origin of this
449 unusual human strain (51). Around the same time, a second human SA11-like RVA strain
450 ZTR-5 was deposited in GenBank by researchers from China as a potential vaccine
451 candidate. However, the controversy about the origin of these SA11-like strains remained.
452 To our surprise, the purple genotype constellation described in this paper, and containing
453 only the bat RVA strains from Gabon, showed a remarkable similarity with these SA11-
454 like strains, sharing up to 9 genotypes with B10 and up to 7 genotypes with SA11 and
455 ZTR-5 (Table 3). Also phylogenetically, these bat RVA strains are the closest relatives of
456 these SA11 like RVA strains for most gene segments (Figures 2-4). The finding that the
457 purple SA11-like genotype constellation was found in multiple bats in Gabon, and only on
458 a single occasion in vervet monkeys and in 2 unrelated human cases, makes bats the
459 prime suspect of being the major hosts of these viruses, making the monkey and humans
460 strains likely examples of interspecies transmissions. It should however be noted that the
461 phylogenetic clustering between these bat, simian and human strains is still rather variable
462 and not as high as was the case between bat RVA strains and E3198 (Table S4, Figures
463 2-4). However, 2 other bat strains are of further interest: 1) the bat RVA strain 322/Kwale
464 (only available as a GenBank entry at this point) seems to have a mixed genotype
465 constellation possessing both characteristics of the orange and purple genotype
466 constellations (Table 3). Especially, the purple genotypes R8, M5 and A5 of 322/Kwale
467 are of interest as they are much more closely related to the SA11-like strain than the
468 Gabon bat RVA strain (Figure 2); 2) the bat RVA strain BATp39 (only available in

GenBank) possesses a single purple genotype I16, and again this is more closely related to the SA11-like strain B10, compared to the Gabon RVA strains. Taken all together, we speculate that with further RVA screenings in bat populations, more bat RVA strains that are closely related to the vervet monkey RVA strain SA11 and human SA11-like RVA strains will be detected.

Further examples of zoonotic transmission to humans

The G3 genotype is usually associated with P[8] genotype in humans RVAs, and combinations such as G3P[3] and G3P[9] are only sporadically found in the human population (70). Nonetheless, in the 2000-2001 season, a rare G3P[3] human rotavirus CMH222 was detected in a 2 year-old severely diarrheic patient in Thailand (41). It was reported to have a VP7 gene closely related to the simian RRV strain and a VP4 gene that was caprine-like. Following this study, Xia and colleagues speculated that even though only the VP4, VP7, VP6 and NSP4 gene segments are characterized, this strain is distinct from typical human RVA genotype constellations and very likely shared a common ancestor with Asian bat RVAs (33). Our current study further adds evidence to the hypothesis of the bat origin of CMH222, as the VP6 I8 genotype of CMH222 is closely related to the GKS-897 strain (Figure 3).

Later on, Wang and colleagues contributed to the list of unusual Southeast Asian human RVA strains. Possessing the G3P[9] genotypes, both the L621 and E2451 strains were isolated from a symptomatic adult and a symptomatic child, in 2006 and 2011, respectively (71). Complete genome analyses revealed a high genetic relatedness to strains of feline/canine origin for almost all 11 genes. L621 and E2451 also clustered near the aforementioned unusual equine strain E3198 for the VP3, VP6, NSP2, NSP5 genes; and

493 L621 additionally also clustered with the E3198 NSP3 gene. In the current study, we have
494 observed that these atypical Asian human strains were also closely related to the
495 Bulgarian bat RVA strains for VP3, VP6, NSP2, NSP4, NSP5 and Gabonese strains for
496 NSP2, NSP3, NSP4 of the orange genotype constellation (Figures 2-4). These additional
497 findings further add, as well as complicate the identification of the most likely bat host,
498 from which the L621 and E2451 strains jumped to humans.
499 Following these potential zoonosis reports, Esona and colleagues also revealed
500 remarkable findings in Latin America in 2018, where only limited bat RVA information is
501 present to date (40). A human RVA strain 2014735512 was isolated in Suriname in 2013,
502 and possessed a rare G20 genotype, which was also detected in an Ecuadorian human
503 RVA strain in 2006. Remarkably, it was associated to the Brazilian bat strain 3081 for
504 VP7, NSP3 and NSP5 segments and speculated to be of bat origin as these genotypes
505 have not been detected in any other animal species so far. The Costa Rican bat RVA
506 strain KCR10-93, which was isolated in this study, also showed a close relatedness to
507 2014735512 strain, and this clearly suggests that this unusual human strain could be of
508 bat RVA origin.
509

510 **Conclusion**

511 Despite the limited number of bat species that have been screened for rotaviruses, a
512 surprisingly large genetic diversity of RVA strains is presented in this study. With
513 increasing screening efforts, it is without a doubt that this diversity will expand both
514 genetically and geographically. We also presented multiple examples of interspecies
515 transmission events involving humans and animals. This has always been restricted to
516 sporadic cases so far and has, to the best of our knowledge, never resulted in major

outbreaks in human. However, it is believed that the rotavirus genotype constellations currently circulating in humans also have a common ancestor with animal rotaviruses (3), highlighting that interspecies transmissions following establishment in the human population could happen again.

Another notable finding is that the SA11 RVA strain, which is used in global rotavirus research for decades, might be of bat origin. Furthermore, this SA11 strain has been recently used as the backbone strain for a RVA reverse genetics system, and is therefore likely to be used even more in the future. It would be intriguing to test whether or not SA11 grows well in bat cell lines, or in *in vivo* infection experiments.

MATERIALS AND METHODS

Sample collection

Faecal samples were collected from 2,142 bats from 10 bat families, representing 46 bat species (Table S2). Sample collection took place in Ghana, Gabon, Bulgaria, Romania, Germany and Costa Rica during 2008-2010 as part of investigations of various other viruses in bats, such as coronavirus, astrovirus, and picornavirus, as described previously (41–45). Bat species were determined by trained field biologists. For European and Costa Rican studies, bats were caught with mist nets, put into cotton bags and faecal pellets are collected. Ghanaian faecal droppings were collected with plastic foil from the trees in which *E. helvum* bats were roosting. The pellets were kept in RNAlater RNA stabilization solution (QIAGEN, Hilden, Germany). Gabonese bats were also captured with mist nets just before twilight and were individually euthanized. Bat faeces were collected with the corresponding permissions in all of the studies according the host countries.

RT-PCR rotavirus screening and viral metagenomics

Viral RNA was isolated from the faecal specimens as described previously (44). To screen the RVA presence in bats, conserved primer pairs targeting the VP1 gene were used (277 nucleotide long PCR product) in a hemi-nested and single round reverse transcription (RT-PCR) assay (Table S1). Among the 18 positive specimens (Tables S2-S3), 16 faecal samples, of which sufficient material was left, were shipped to the Laboratory of Clinical and Epidemiological Virology, Leuven, Belgium on dry ice for further complete genome analyses (Table 1).

The NetoVIR protocol was used for viral enrichment of the faecal suspensions as described before (72). Briefly, the faecal samples were suspended in dPBS and homogenized with a MINILYS homogenizer (Bertin Technologies) for 20s at 3,000 rpm. The homogenates were centrifuged for 3 min at 17,000 g and filtered with 0,8 µm PES filters (Sartorius). Filtrates were treated with benzonase (Novagen) and micrococcal nuclease (New England Biolabs) at 37 °C for 2 h to remove the free-floating nucleic acids. Subsequently, samples were extracted using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions, without addition of carrier RNA to the lysis buffer. Reverse transcription and second strand synthesis was performed by an adjusted version of the Whole Transcriptome Amplification (WTA2) protocol as described previously (Sigma-Aldrich) (73). Sequencing library was constructed with the Nextera XT Library Preparation Kit (Illumina). The size of the library was checked with Bioanalyzer (Agilent Technologies) with a High Sensitivity DNA chip and the 2nM pooled libraries were sequenced on an Illumina NextSeq 500 platform (2x150bp paired-end).

Data analysis

Low quality reads, ambiguous bases, primer and adapter sequences were removed from the paired-end reads with Trimmomatic v0.36 with default parameters (74). Trimmed reads were *de novo* assembled with metaSPAdes from SPAdes software v3.11.1 using 21, 33, 55, 77 k-mer lengths (75). The obtained contigs were annotated with DIAMOND v0.9.10 against a non-redundant protein database (76). The contigs annotated as "Rotavirus" were further investigated using the nucleotide BLAST against a nucleotide reference database to identify the gene segments (77). The incomplete contigs were completed *in silico* by mapping the trimmed reads of corresponding samples against the reference sequence determined by the highest BLASTn nucleotide similarity with the lowest e-value using BWA software v0.5.9 (78) and SAMtools v1.6 (79). Open reading frames were determined by the web-based NCBI ORF Finder tool (80) (www.ncbi.nlm.nih.gov/orffinder).

Genotype constellations and phylogenetic analyses

The genotypes were assigned using RotaC tool (http://rotac.regatools.be). The sequences that could not be assigned to any established genotype were sent to the RCWG for assignment of novel genotypes.

Codon-based nucleotide level multiple sequence alignments were done using MUSCLE (81) with default parameters in MEGA software v7.0.26 (82). Pairwise nucleotide distances were calculated using maximum composite likelihood algorithm (83). Alignments were trimmed with trimAL v1.2 and GTR+G+I substitution model was used (84). Phylogenetic trees were reconstructed with BEAST software v1.10.4 (85). The BEAST input file was configured in BEAUTi with strict molecular clock and constant size

589 coalescent tree prior. 100,000,000 sample states were generated in MCMC analysis at
590 every 10,000-50,000 steps with effective sample size (ESS) values for all the continuous
591 parameters higher than 200, and posterior probabilities were calculated. The initial 10%
592 of the sample trees were discarded as burn-in in TreeAnnotator v1.8.4 (86). Tracer v1.6
593 (87) was used for the visualization of the MCMC trace files and FigTree v1.4.3 from the
594 BEAST package was used for phylogenetic tree visualization and manipulation (88).
595 Maximum likelihood trees with 500 bootstraps and Tamure-Nei model were also
596 generated in MEGA to confirm the tree topologies. The genotype constellations are
597 illustrated on a world map using the maps package in R software (89).
598
599 **Data availability**
600 The data have been deposited with links to BioProject accession number
601 PRJNA562472 in the NCBI BioProject database
602 (https://www.ncbi.nlm.nih.gov/bioproject/). The data is also deposited to GenBank under
603 the following accession numbers: MN433617-27 (BB89-15), MN539284-94 (BR89-60),
604 MN528116-26 (GKS-897), MN477236-46 (GKS-912), MN528101-15 (GKS-926),
605 MN528075-85 (GKS-929), MN528086-MN528100 (GKS-934), MN551587-97 (GKS-941),
606 MN477225-35 (GKS-954), MN551598-MN551608 (KCR10-93), MN567261-72 (K212).
607
608 **Ethical Statement**
609 Bat capture and sampling were conducted with the permissions of the Wildlife and Hunting
610 Department of the Gabonese Ministry of Water and Forestry (N°003/MEFE-PA/
611 SG/DGEF/DCF and N°0021/MEFE-PA/SG/DGEF/DCF), and under clearance
612 314/5327.74.1.6 from the State Office of Energy and Agriculture, the Environment and

Rural Areas Schleswig-Holstein (LANU) and clearances 133/24.03.2008 and 192/ 26.03.2009 from the Bulgarian Ministry of Environment and Water. For the Ghanaian bats, ethics approval was obtained from the Committee for Human Research, Publications and Ethics of Komfo Anokye Teaching Hospital and School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi. Research samples were exported under a state agreement between the Republic of Ghana and the Federal Republic of Germany, represented by the City of Hamburg. Additional export permission was obtained from the Veterinary Services of the Ghana Ministry of Food and Agriculture.

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943

944 **Tables and Figures**

945 **Table 1.** Meta-data and NGS summary of the sequenced RVA-positive samples

Sample ID	Location	Country	Year	Bat species	Bat Diet	Raw Reads	Trimmed Reads	N° of RVA reads[a]	RVA read percentage[b]
BB89-15	Elenas Cave	Bulgaria	2008	*Rhinolophus blasii*	Insect	13,508,743	3,850,458	56,536	1.5%
BR89-60	Roman Horse Cave	Bulgaria	2008	*Rhinolophus euryale*	Insect	11,812,353	3,224,700	2,278	0.1%
SW78-39	Wahlstorf, SH	Germany	2008	*Myotis daubentonii*	Insect	5,720,709	5,411,241	0	0.0%
GKS-660	Zadie	Gabon	2009	*Hipposideros caffer*	Insect	7,356,697	5,404,115	4	0.0%
GKS-897	Faucon	Gabon	2009	*Hipposideros gigas*	Insect	6,994,665	3,938,299	30,929	0.8%
GKS-912	Faucon	Gabon	2009	*Hipposideros gigas*	Insect	4,018,151	2,968,694	1,236,102	41.6%
GKS-926	Faucon	Gabon	2009	*Hipposideros gigas*	Insect	6,346,691	4,955,591	4,479,073	90.4%
GKS-929	Faucon	Gabon	2009	*Hipposideros gigas*	Insect	993,739	718,192	315,056	43.9%
GKS-934	Faucon	Gabon	2009	*Hipposideros gigas*	Insect	7,341,726	5,454,901	35,259	0.7%
GKS-941	Faucon	Gabon	2009	*Hipposideros gigas*	Insect	5,923,863	3,741,568	442,380	11.8%
GKS-942	Faucon	Gabon	2009	*Hipposideros gigas*	Insect	8,363,558	6,453,805	0	0.0%
GKS-953	Faucon	Gabon	2009	*Hipposideros gigas*	Insect	4,361,523	3,358,374	22	0.0%
GKS-954	Faucon	Gabon	2009	*Hipposideros gigas*	Insect	7,358,552	5,683,659	201,335	3.5%
GKS-955	Faucon	Gabon	2009	*Hipposideros gigas*	Insect	5,704,559	3,820,529	23	0.0%
K212	Kumasi	Ghana	2009	*Eidolon helvum*	Fruit	8,367,278	5,189,608	17,206	0.3%
KCR10-93	Orosi	Costa Rica	2010	*Carollia perspicillata*	Insect	7,731,234	2,235,422	12,179	0.5%
Average						6,994,003	4,150,572	426,774	12.2%
Total						118,929,778	67,370,384	6,828,382	

[a] Number of unique trimmed reads mapping to RVA genomic segments in the corresponding sample

[b] Proportion of RVA reads to all the reads in the corresponding sample

946

947 **Table 2.** Color-coded genotype constellations of the bat RVA strains identified in this
948 study. In some samples, 2 different variants of the same gene segments were identified,
949 suggesting co-infections. K212 possessed 2 distinct VP3 gene segments belonging to the
950 same M14 genotype (indicated with an asterisk). NSP5 gene of KCR10-93 could not be
951 assigned to any of the established genotypes; neither assigned to a novel genotype as
952 the complete ORF could not be determined. Therefore, this genotype is indicated as “H?”.
953

Strains	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Bat-wt/BGR/BB89-15/2008/G3P[3]	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Bat-wt/BGR/BB89-60/2008/G3P[3]	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Bat-wt/GAB/GKS-897/2009/G3P[3]	G3	P[3]	I8	R8	C5	M5	A5	N3	T5	E3	H5
RVA/Bat-wt/GAB/GKS-954/2009/G3P[3]	G3	P[3]	I16	R8	C5	M5	A5	N3	T3	E3	H5
RVA/Bat-wt/GAB/GKS-941/2009/G3P[3]	G3	P[3]	I16	R8	C5	M5	A5	N3	T3	E3	H5
RVA/Bat-wt/GAB/GKS-929/2009/G3P[2]	G3	P[2]	I16	R8	C5	M5	A5	**N23**	T5	**E28**	H5
RVA/Bat-wt/GAB/GKS-912/2009/G3P[3-2]	G3	P[3] P[2]	I16	R8	C5	M5	A5	N3	T3	E3	H5
RVA/Bat-wt/GAB/GKS-926/2009/G3P[3-2]	G3	P[3] P[2]	I16	R8	C5	M5	A5	N3 **N23**	T3 T5	E3 **E28**	H5
RVA/Bat-wt/GAB/GKS-934/2009/G3P[3-2]	G3	P[2] P[3]	I16	R8	C3 C5	M3 M5	A5	N3	T5 T3	E3	H5
RVA/Bat-wt/GHA/K212/2009/G30P[47]	G30	P[47]	I22	R15	C15	M14*	A25	N15	T17	E22	H17
RVA/Bat-wt/CRC/KCR10-93/2010/G20P[47]	G20	P[47]	I13	R13	C13	M12	**A32**	N13	**T23**	E20	**H?**

954 **Table 3.** Colour-coded genotype constellations for the bat RVA strains identified in this
955 study, previously published bat RVA strains, as well as a selection of RVA strains from
956 other host species potentially related to bats. The non-sequenced segments or
957 unassigned genotypes are denoted with '[letter code]?'. The genotypes coloured in light
958 grey are less relevant due to a lack of (in)direct genomic relationship with bat RVAs
959 identified in the current study. The strain names are colour-matched with the
960 corresponding genotype constellations (orange, purple, blue, green, brown, grey and
961 yellow).

962

Strains	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-tc/JPN/AU-1/1982/G3P[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Human-wt/CHN/E2451/2011/G3P[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human-tc/CHN/L621/2006/G3P[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Horse-wt/ARG/E3198/2008/G3P[3]	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Bat-wt/BGR/BB89-15/2008/G3P[3]	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Bat-wt/BGR/BB89-60/2008/G3P[3]	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Bat-wt/CHN/LZHP2/2015/G3P[3]	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Bat-wt/BRA/4754/2013/G3P[3]	G3	P[3]	I?	R?	C?	M?	A?	N?	T3	E3	H6
RVA/Bat-tc/CHN/MSLH14/2012/G3P[3]	G3	P[3]	I8	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Bat-wt/CHN/BSTM70/2015/G3P[3]	G3	P[3]	I8	R3	C3	M3	A29	N3	T3	E3	H6
RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	G3	P[10]	I8	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Human-wt/US/09US7118/2009/G3P[24]	G3	P[24]	I2	R2	C3	M3	A9	N3	T3	E3	H6
RVA/Monkey/USA/TUCH/2003/G3P[24]	G3	P[24]	I9	R3	C3	M3	A9	N1	T3	E3	H6
RVA/Simian-tc/USA/RRV/1975/G3P[3]	G3	P[3]	I9	R2	C3	M3	A9	N2	T3	E3	H6
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]	G3	P[3]	I3	R2	C2	M3	A9	N2	T3	E2	H3
RVA/Dog-tc/ITA/RV198-95/1995/G3P[3]	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog/HUN/135/2012/G3P[3]	G3	P[3]	I3	R3	C3	M3	A15	N2	T3	E3	H6
RVA/Bat-wt/CHN/YSSK5/2015/G3P[3]	G3	P[3]	I8	R20	C2	M1	A9	N3	T3	E3	H6
RVA/Bat/KEN/322/Kwale/2015/G3P[10]	G3	P[10]	I2	R8	C3	M5	A5	N3	T6	E3	H6
RVA/Bat-wt/GAB/GKS-897/2009/G3P[3]	G3	P[3]	I8	R8	C5	M5	A5	N3	T5	E3	H5
RVA/Bat-wt/GAB/GKS-954/2009/G3P[3]	G3	P[3]	I16	R8	C5	M5	A5	N3	T3	E3	H5
RVA/Bat-wt/GAB/GKS-941/2009/G3P[3]	G3	P[3]	I16	R8	C5	M5	A5	N3	T3	E3	H5
RVA/Bat-wt/GAB/GKS-929/2009/G3P[2]	G3	P[2]	I16	R8	C5	M5	A5	**N23**	T5	**E28**	H5
RVA/Bat-wt/GAB/GKS-912/2009/G3P[3-2]	G3	P[3] P[2]	I16	R8	C5	M5	A5	N3	T3	E3	H5
RVA/Bat-wt/GAB/GKS-926/2009/G3P[3-2]	G3	P[3] P[2]	I16	R8	C5	M5	A5	N3 **N23**	T3 T5	E3 **E28**	H5
RVA/Bat-wt/GAB/GKS-934/2009/G3P[3-2]	G3	P[2] P[3]	I16	R8	C3 C5	M3 M5	A5	N3	T5 T3	E3	H5
RVA/Human-tc/KEN/B10/1987/G3P[2]	G3	P[2]	I16	R8	C5	M5	A5	N5	T5	E13	H5
RVA/Simian-tc/ZAF/SA11-N2/1958/G3P[2]	G3	P[2]	I2	R2	C5	M5	A5	N5	T5	E2	H5
RVA/Human/CHN/ZTR-5/G3P[2]	G3	P[2]	I2	R2	C5	M5	A5	N2	T5	E2	H5
RVA/Bat-wt/KEN/BATp39/2015/G36P[51]	G36	P[51]	I16	R22	C20	M20	A31	N22	T22	E27	H22
RVA/Bat-wt/CMR/BatLy17/2014/G30P[47]	G30	P[47]	I22	R15	C15	M14	A25	N15	T17	E22	H17
RVA/Bat-wt/GHA/K212/2009/G30P[47]	G30	P[47]	I22	R15	C15	M14 M14	A25	N15	T17	E22	H17
RVA/Bat-wt/CMR/BatLi10/2014/G30P[42]	G30	P[42]	I22	R15	C15	M14	A25	N15	T17	E22	H17
RVA/Bat-wt/CMR/BatLi09/2014/G30P[42]	G30	P[42]	I22	R15	C15	M14	A25	N15	T17	E22	H17
RVA/Bat-wt/CMR/BatLi08/2014/G31P[42]	G31	P[42]	I22	R15	C15	M14	A25	N15	T17	E22	H17
RVA/Bat-wt/ZMB/ZFB14-52/2014/G31P[x]	G31	P?	I22	R?	C?	M?	A?	N?	T17	E?	H?
RVA/Bat-wt/ZMB/ZFB14-135/2014/G31P[x]	G31	P?	I22	R15	C?	M?	A?	N?	T17	E?	H?
RVA/Bat-wt/ZMB/ZFB14-126/2014/GxP[x]		P?	I22		C?	M?	A?	N21	T17	E27	H?
RVA/Bat-wt/CRC/KCR10-93/2010/G20P[47]	G20	P[47]	I13	R13	C13	M12	**A32**	N13	**T23**	E20	**H?**
RVA/Bat-wt/3081/BRA/2013/G20P[x]	G20	P?	I?	R?	C?	M?	A?	N?	T15	E?	H15
RVA/Human-wt/SUR/2014735512/2013/G20P[28]	G20	P[28]	I13	R13	C13	M12	A23	N13	T15	E20	H15
RVA/Human-wt/ECU/Ecu534/2006/G20P[28]	G20	P[28]	I13	R?	C?	M?	A?	N?	T?	E?	H?
RVA/Bat-wt/CHN/GLRL1/2005/G33P[48]	G33	P[48]	I25	R19	C18	M18	A?	N19	T20	E25	H20
RVA/Bat-wt/CMR/BatLy03/2014/G25P[43]	G25	P[43]	I15	R16	C8	M15	A26	N8	T11	E23	H10
RVA/Bat/SAU/KSA402/2012/G25P[43]	G25	P[43]	I15	R16	C8	M15	A26	N8	T11	E23	H10
RVA/Bat-wt/KEN/KE4852/07/2007/G25P[6]	G25	P[6]	I15	R?	C8	M?	A?	N8	T11	E2	H10

963

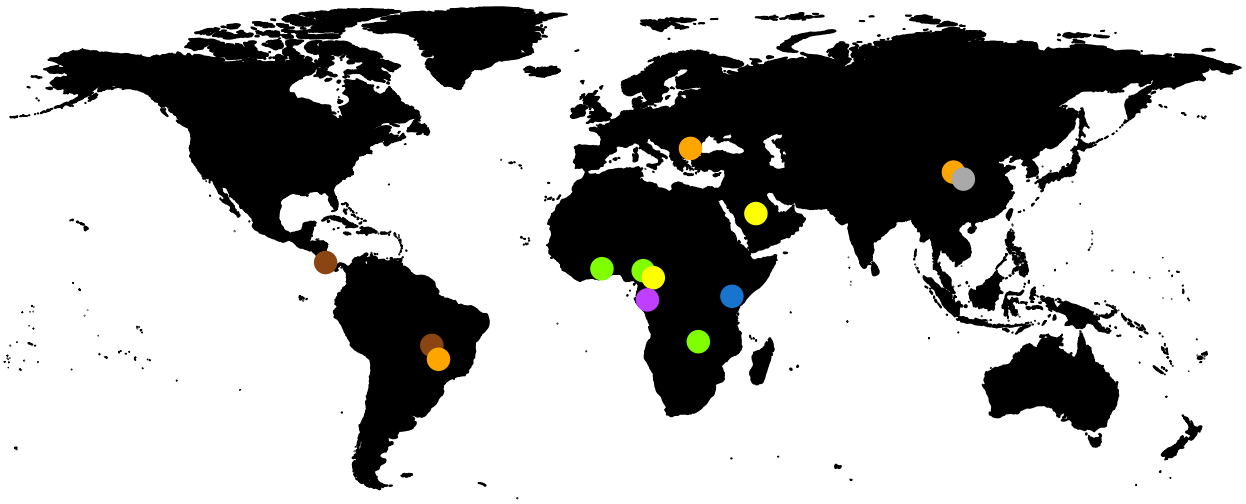
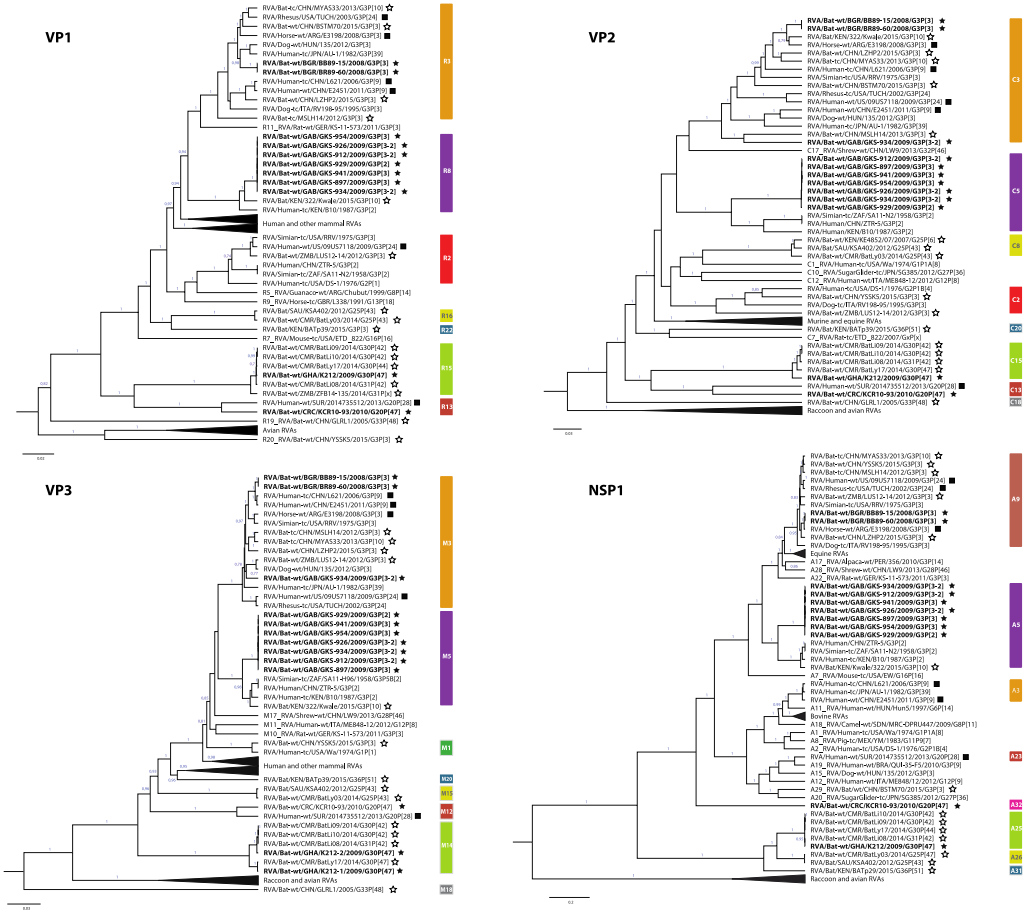


Figure 1. Geographic distribution of the currently known bat RVA genotype constellations. The coloured circles represent the circulating genotypes at the specified locations according to the strain colours highlighted in Table 3.

967



968

969 **Figure 2**. Maximum clade credibility trees of the VP1, VP2, VP3 and NSP1 gene
970 segments of the identified bat RVA strains with known human and other mammal RVAs
971 using BEAST. Only posterior probability values above 0.7 are shown. The genotypes of
972 the strains are listed on the right side of the trees. The bat RVA strains identified in this
973 study are shown in bold marked with filled stars, previously reported bat RVA strains are
974 marked with open stars, and non-bat RVA strains related to a bat RVA strain are marked
975 with filled squares.

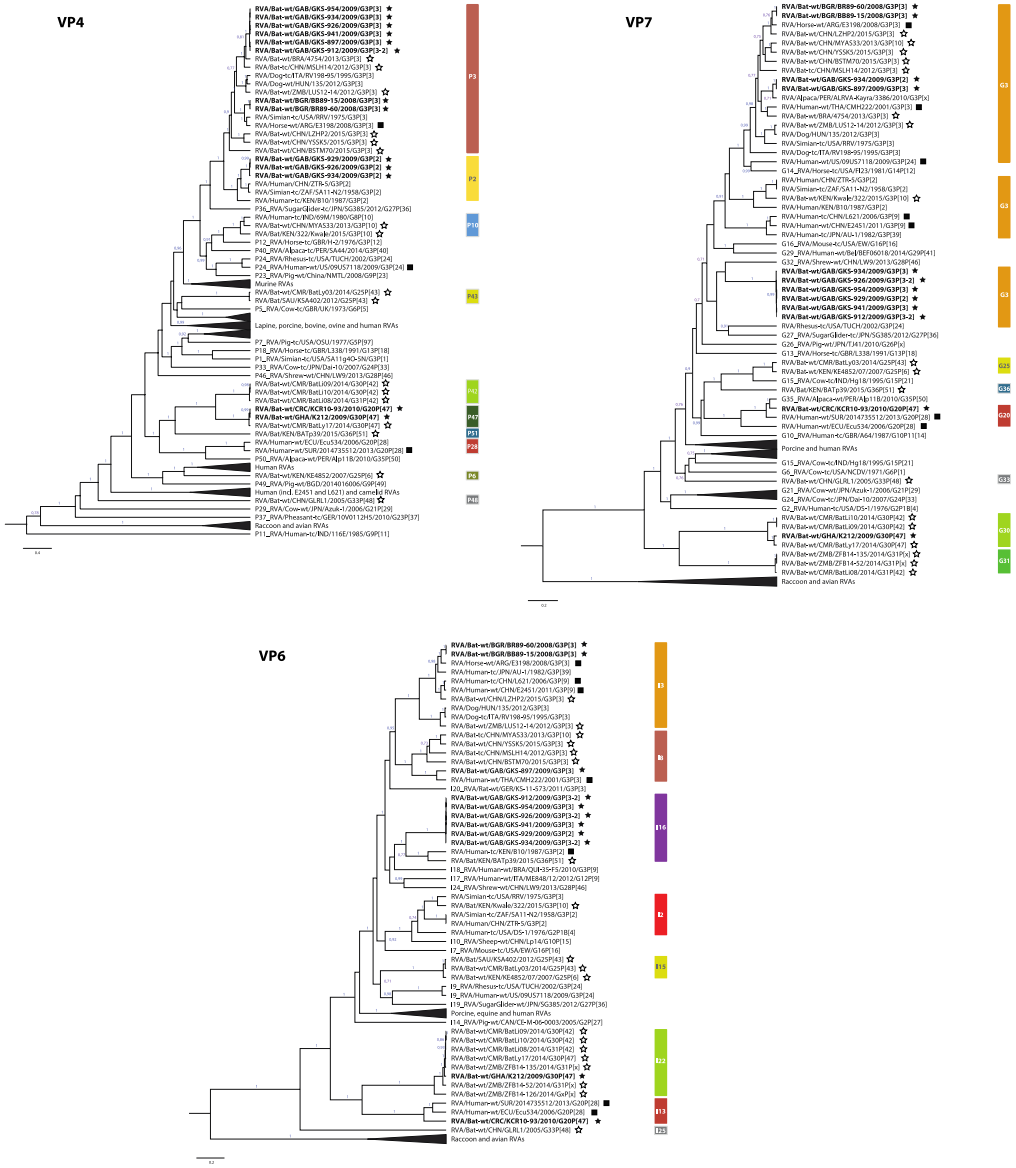


Figure 3. Maximum clade credibility trees of the VP4, VP6 and VP7 gene segments of the identified bat RVA strains with known human and other mammal RVAs using BEAST. Only posterior probability values above 0.7 are shown. The genotypes of the strains are listed on the right side of the trees. The bat RVA strains identified in this study are shown in bold marked with filled stars, previously reported bat RVA strains are marked with open stars, and non-bat RVA strains related to a bat RVA are marked with filled squares.

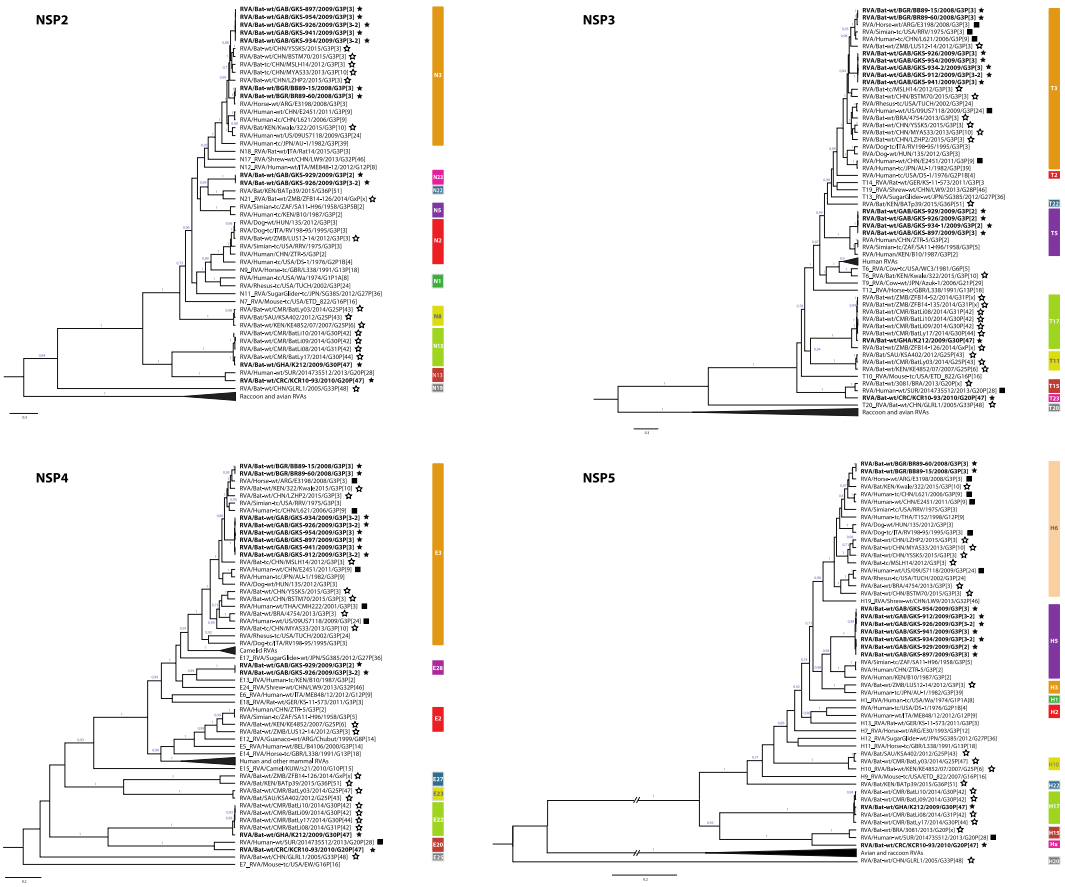


Figure 4. Maximum clade credibility trees of the NSP2, NSP3, NSP4 and NSP5 gene segments of the identified bat RVA strains with known human and other mammal RVAs using BEAST. Only posterior probability values above 0.7 are shown. The genotypes of the strains are listed on the right side of the trees. The bat RVA strains identified in this study are shown in bold marked with filled stars, previously reported bat RVA strains are marked with open stars, and non-bat RVA strains related to a bat RVA strain are marked with filled squares.

993 **Table S1.** RT-PCR oligonucleotides for the initial rotavirus screening against VP1

ID no.	Sequence (5' → 3')	Position	Genome segment	Polarity	Assay type
PanRota-F1570	TAYACIGAYGTITCICARTGGGA	1570-1593[a]	VP1	+	Heminested RT-PCR, 1st round
PanRota-R1922	GCGTAGTTGTCGTCICCRTCBAC	1900-1922[a]	VP1	-	1st and 2nd rd
PanRota-F1585a	CARTGGGATTCGTCICAGCAYAAYAC	1585-1610[a]	VP1	+	2nd rd
PanRota-F1585b	CARTGGGACGCCAGICAACATAAYAC	1585-1610[a]	VP1	+	2nd rd

994

995 ID, identification; RT-PCR, reverse transcription–PCR; [a]corresponding to Rotavirus A
996 G11P[25] Dhaka6 VP1 (GenBank # EF560705); Variant forms of primers (marked
997 consecutively with an alphabetic character in the last position) were mixed together
998 equally and from then on treated as one single primer.

999 **Table S2.** Taxonomical annotation, sampling time and location, RVA PCR detection of

1000 the bat samples

Order-Family	Species	No. of samples per sampling site and year								PCR positive (%)	Positive samples (ID)
		total	BGR 2008	BGR 2009	CRC 2010	GAB 2009	DEU 2008	GHA 2009	ROU 2008		
Chiroptera-Pteropodidae	*Eidolon helvum*	**226**						226		1 (0.4%)	K212
	Micropteropus pusillus	**1**						1		0 (0%)	
	Rousettus aegyptiacus	**10**				8		2		0 (0%)	
Chiroptera-Rhinolophidae	*Rhinolophus blasii*	**90**	82	8						1 (1.1%)	BB89-15
	Rhinolophus euryale	**336**	244	92						2 (0.6%)	BBR89-2, BR89-60
	Rhinolophus ferrum-equinum	**52**	45	6					1	0 (0%)	
	Rhinolophus hipposideros	**6**	6								
	Rhinolophus landeri	**1**						1		0 (0%)	
	Rhinolophus mehelyi	**22**	14	8						0 (0%)	
	Rhinolophus spec.	**6**				6				0 (0%)	
Chiroptera-Hipposideridae	*Hipposideros cf ruber/caffer*	**183**				46		137		2 (1.1%)	GKS-637, GKS-660
	Hipposideros cf spec	**2**						2		0 (0%)	
	Hipposideros gigas	**67**				67				10 (14.9%)	GKS-897, GKS-912, GKS-926, GKS-929, GKS-934, GKS-941, GKS-942, GKS-953, GKS-954, GKS-955
	Hipposideros abae	**62**						62		0 (0%)	
Chiroptera-Nycteridae	*Nycteris spec.*	**3**						3		0 (0%)	
Chiroptera-Emballonuridae	*Coleura afra*	**5**						5		0 (0%)	
	Peropteryx kappleri	**5**			5					0 (0%)	
Chiroptera-Phyllostomidae	*Anoura geoffroyi*	**100**			100					0 (0%)	
	Carollia castanea	**1**			1					0 (0%)	
	Carollia perspicillata	**203**			203					1 (0.5 %)	KCR10-93
	Enchisthenes hartii	**3**			3					0 (0%)	
	Glossophaga commissarisi	**3**			3					0 (0%)	
	Glossophaga soricina	**22**			22					0 (0%)	
Chiroptera-Mormoopidae	*Pteronotus parnellii*	**21**			21					0 (0%)	
Chiroptera-Natalidae	*Natalus lanatus*	**3**			3					0 (0%)	
Chiroptera-Vespertilionidae	*Barbastella barbastellus*	**13**	12						1	0 (0%)	
	Miniopterus inflatus	**2**				2				0 (0%)	
	Miniopterus schreibersii	**77**	39						38	0 (0%)	
	Myotis brandtii	**17**					17			0 (0%)	
	Myotis alcathoe	**2**	2							0 (0%)	
	Myotis bechsteinii	**57**	32				25			0 (0%)	
	Myotis capaccini	**1**	1							0 (0%)	
	Myotis dasycneme	**149**					149			0 (0%)	
	Myotis daubentonii	**110**	7				103			1 (0.9%)	SW78-39
	Myotis emarginatus	**5**	5							0 (0%)	
	Myotis myotis	**77**	3				60		14	0 (0%)	
	Myotis mystacinus	**51**					51			0 (0%)	
	Myotis nattereri	**27**	2				25			0 (0%)	
	Myotis oxygnathus	**22**	1						21	0 (0%)	
	Nyctalus leisleri	**3**	3							0 (0%)	
	Nyctalus noctula	**11**					2		9	0 (0%)	
	Pipistrellus cf nanus/nanulus	**3**						3		0 (0%)	

	Pipistrellus nathusii	**2**					2			0 (0%)	
	Pipistrellus pipistrellus	**37**					37			0 (0%)	
	Pipistrellus pygmaeus	**29**	2				27			0 (0%)	
	Pipistrellus spec.	**6**						6		0 (0%)	
	Plecotus auritus	**5**	2				3			0 (0%)	
	Plecotus austriacus	**1**					1			0 (0%)	
Chiroptera-Molossidae	*Mops spec.*	**2**		1				1		0 (0%)	
	Total (46 species)	**2142**	**502**	**115**	**361**	**129**	**502**	**449**	**84**	**18 (0.8%)**	

Country: BGR = Bulgaria; CRC = Costa Rica; GAB = Gabon; DEU = Germany; GHA = Ghana; ROU = Romania

1003 **Table S3.** RVA-positive bat samples detected by targeted RT-PCR and undergone viral

1004 metagenomics

Sample	Host	Country	Place	Year
BBR89-2	*Rhinolophus euryale*	Bulgaria	Bratanova	2008
BB89-15	*Rhinolophus blasii*		Elenas Cave	
BR89-60	*Rhinolophus euryale*		Roman Horse Cave	
SW78-39	*Myotis daubentonii*	Germany	Wahlstorf, SH	2008
GKS-660	*Hipposideros caffer*	Gabon	Zadie	2009
GKS-637				
GKS-897	*Hipposideros gigas*	Gabon	Faucon	2009
GKS-912				
GKS-926				
GKS-929				
GKS-934				
GKS-941				
GKS-942				
GKS-953				
GKS-954				
GKS-955				
K212	*Eidolon helvum*	Ghana	Kumasi	2009
KCR10-93	*Carollia perspicillata*	Costa Rica	Orosi	2010

1005

1006 **Table S4.** a. Examples of reassortments among bat RVA strains. b. Examples of distinct
1007 RVA genotype constellations in the same bat species. c. Examples of bat RVA strains
1008 with unusual genotype constellations, potentially resulting from (multiple) reassortment
1009 events
1010

a. Strains	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	Host Species	Host Family	Diet
RVA/Bat-wt/BGR/BB89-15/2008/G3P[3]	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6	*Rhinolophus blasii*	*Rhinolophidae*	I
RVA/Bat-wt/BGR/BB89-60/2008/G3P[3]	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6	*Rhinolophus euryale*	*Rhinolophidae*	I
RVA/Bat-wt/CHN/LZHP2/2015/G3P[3]	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6	*Hipposideros pomona*	*Hipposideridae*	I
RVA/Bat-tc/CHN/MSLH14/2012/G3P[3]	G3	P[3]	I8	R3	C3	M3	A9	N3	T3	E3	H6	*Rhinolophus hipposideros*	*Rhinolophidae*	I
RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	G3	P[10]	I8	R3	C3	M3	A9	N3	T3	E3	H6	*Aselliscus stoliczkanus*	*Hipposideridae*	I
RVA/Bat-wt/CHN/BSTM70/2015/G3P[3]	G3	P[3]	I8	R3	C3	M3	A29	N3	T3	E3	H6	*Taphozous melanopogon*	*Emballonuridae*	I/F
RVA/Bat-wt/CHN/YSSK5/2015/G3P[3]	G3	P[3]	I8	R20	C2	M1	A9	N3	T3	E3	H6	*Scotophilus kuhlii*	*Vespertilionidae*	I

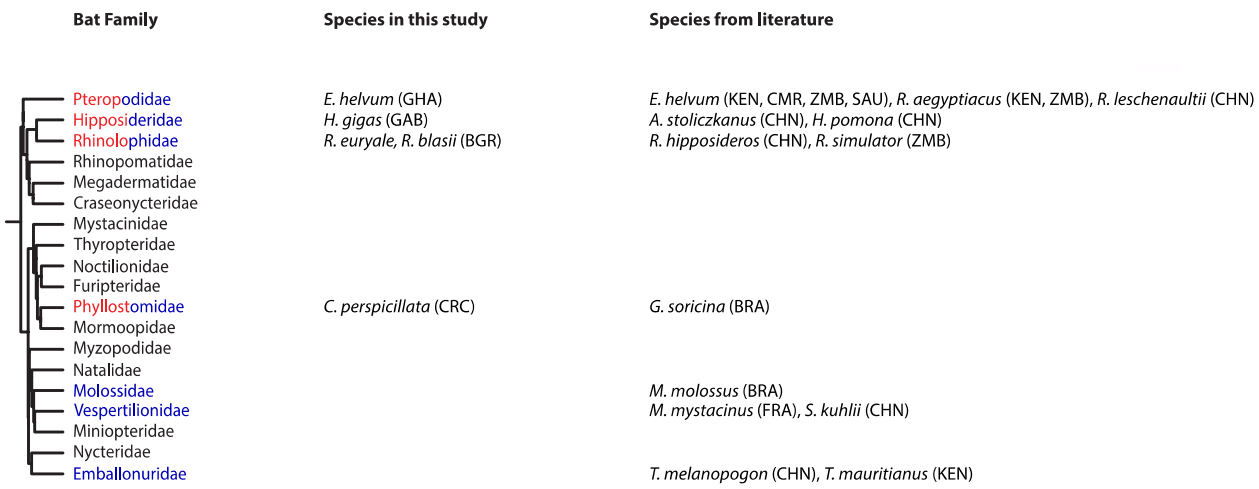
1011

b. Strains	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	Host Species	Host Family	Diet
RVA/Bat-wt/CMR/BatLy17/2014/G30P[47]	G30	P[47]	I22	R15	C15	M14	A25	N15	T17	E22	H17	*Eidolon helvum*	*Pteropodidae*	F
RVA/Bat-wt/GHA/K212/2009/G30P[47]	G30	P[47]	I22	R15	C15	M14	A25	N15	T17	E22	H17	*Eidolon helvum*	*Pteropodidae*	F
RVA/Bat-wt/CMR/BatLy03/2014/G25P[43]	G25	P[43]	I15	R16	C8	M15	A26	N8	T11	E23	H10	*Eidolon helvum*	*Pteropodidae*	F
RVA/Bat/SAU/KSA402/2012/G25P[43]	G25	P[43]	I15	R16	C8	M15	A26	N8	T11	E23	H10	*Eidolon helvum*	*Pteropodidae*	F

1012

c. Strains	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	Host Species	Host Family	Diet
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]	G3	P[3]	I3	R2	C2	M3	A9	N2	T3	E2	H3	*Rhinolophus simulator*	Rhinolophidae	I
RVA/Bat-wt/CHN/YSSK5/2015/G3P[3]	G3	P[3]	I8	R20	C2	M1	A9	N3	T3	E3	H6	*Scotophilus kuhlii*	Vespertilionidae	I
RVA/Bat/KEN/322/Kwale/2015/G3P[10]	G3	P[10]	I2	R8	C3	M5	A5	N3	T6	E3	H6	*Taphozous mauritianus*	Emballonuridae	I

1013



1014

1015 **Figure S1.** RVA-positive bat families and species. The RVA-positive bat families
1016 reported in the present study (red) and in literature (blue) are shown on the phylogenetic
1017 tree adapted from Simmons et al (2003). No RVA is reported in families in black. A family
1018 is accepted positive for the literature group if more than 1 RVA segment was submitted
1019 to GenBank. The corresponding bat species and the country of sample collection are
1020 also displayed. Country: GHA = Ghana, FRA = France, BRA = Brazil, ZMB = Zambia,
1021 SAU = Saudi Arabia, CRC = Costa Rica, KEN = Kenya, CHN = China, BGR = Bulgaria,
1022 GAB = Gabon

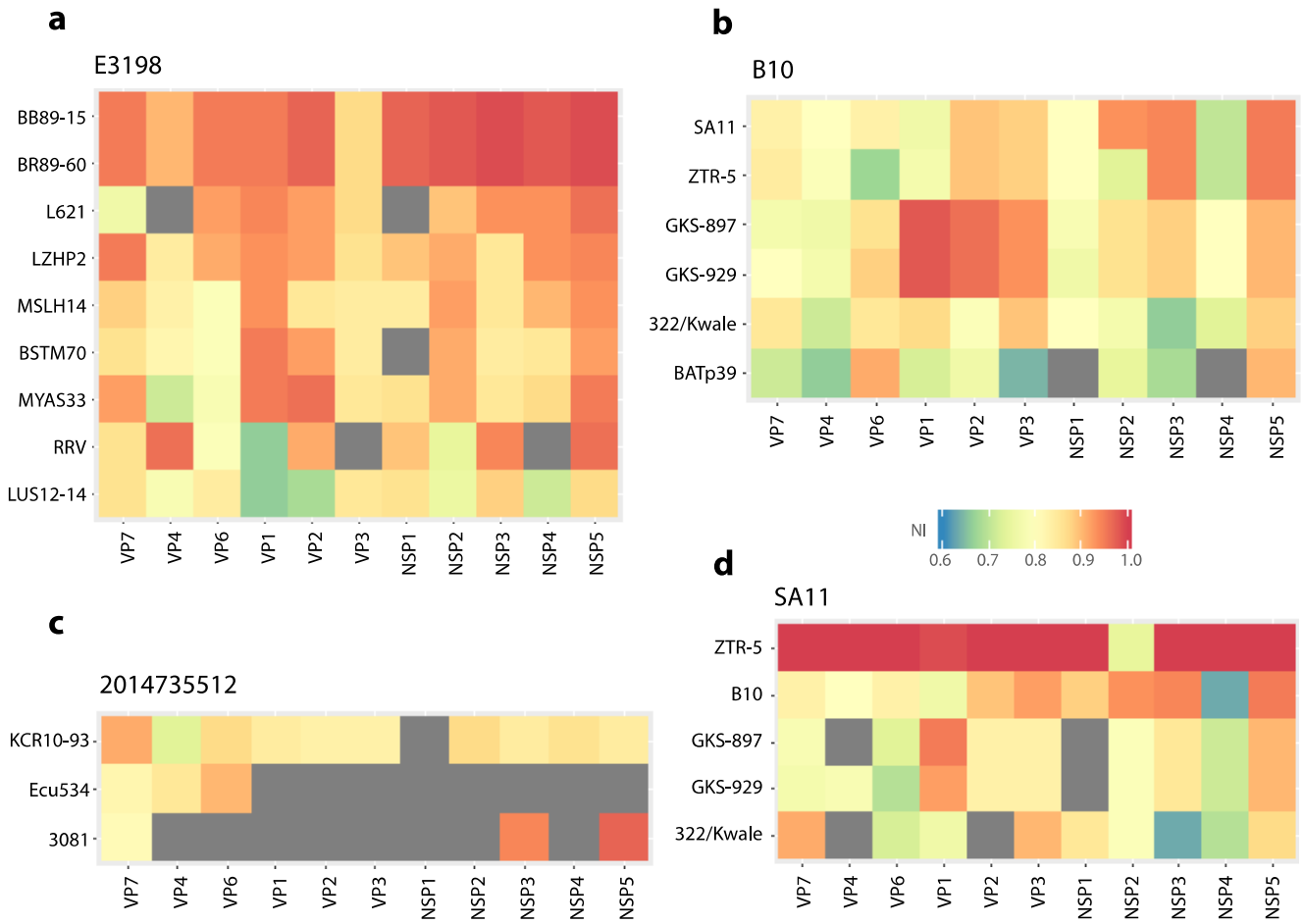


Figure S2. Heatmap of pairwise nucleotide identities (NI) of the unusual RVA strains: E3198 (a), B10 (b), 2014735512 (c), SA11 (d). Grey colour indicates the nucleotide identities below 0.6 or lack of sequence information for the compared strain

Supplementary Material and Methods

Screening VP1-Consensus-PCR

25-µL SuperScript© III with Platinum© Taq DNA Polymerase One-Step RT-PCR reactions as described by the manufacturer (INVITROGEN, Karlsruhe, Germany) used 800 nM each of 1st-round primers, 1 µg bovine serum albumin, $MgSO_4$ up to a total concentration of 2.4 mM, plus 5 µL RNA extract. Amplification involved 30 min at 48°C; 3 min at 95°C; 10 cycles of 20 s at 95°C, 20 s starting at 60°C with a decrease of 1°C per cycle, and 35 s at 72°C; 40 cycles of 20 s at 95°C, 20 s at 50°C, and 35 s at 72°C; and a final elongation step of 2 min at 72°C. 50-µL Platinum Taq reactions as described by the manufacturer (INVITROGEN, Karlsruhe, Germany) used 2 µL of 1st-round PCR product, 2 mM $MgCl_2$ and 800 nM of 2nd-round forward primer and 400 nM of the reverse primer. Amplification involved 3 min at 95°C; 10 cycles of 15 s at 95°C, 15 s starting at 62°C with a decrease of 1°C per cycle, and 30 s at 72°C; 40 cycles 15s at 95°C, 15 s at 52°C and 30 s at 72°C; and a final elongation step of 2 min at 72°C. All PCR reactions were carried out in an Eppendorf Mastercycler ep gradient S (Eppendorf AG, Hamburg, Germany).

Pairwise nucleotide identity heatmap

The calculated pairwise nucleotide identites were represented in heatmaps using the ggplot2 package in R software.