Evaluation of bat adenoviruses suggests co-evolution and host roosting behaviour as drivers for diversity

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Abstract
Adenoviruses (AdVs) are diverse pathogens of humans and animals, with several dozen bat AdVs already identified. Considering that over 100 human AdVs are known, and the huge diversity of bat species, many bat AdVs likely remain undiscovered. To learn more about AdV prevalence, diversity and evolution, we sampled and tested bats in Cameroon using several PCR assays for viral and host DNA. AdV DNA was detected in 14% of the 671 sampled animals belonging to 37 different bat species. There was a correlation between species roosting in larger groups and AdV DNA detection. The detected AdV DNA belonged to between 28 and 44 different, mostly previously unknown, mastadenovirus species. The novel isolates are phylogenetically diverse and while some cluster with known viruses, others appear to form divergent new clusters. The phylogenetic tree of novel and previously known bat AdVs does not mirror that of the various host species, but does contain structures consistent with a degree of virus–host co-evolution. Given that closely related isolates were found in different host species, it seems likely that at least some bat AdVs have jumped species barriers, probably in the more recent past; however, the tree is also consistent with such events having taken place throughout bat AdV evolution. AdV diversity was highest in bat species roosting in large groups. The study significantly increased the diversity of AdVs known to be harboured by bats, and suggests that host behaviours, such as roosting size, may be what limits some AdVs to one species rather than an inability of AdVs to infect other related hosts.

DATA SUMMARY
Ten supplementary data files (Files S1–S10) for this study can be found on Figshare at https://figshare.com/s/31d9a89820f7bcea32c2. Novel viral sequences are listed in File S1 and have been deposited in GenBank with accession numbers MN136540–MN136633. Previously published sequences were downloaded from GenBank, their accession numbers are listed in File S2.

INTRODUCTION
Adenoviruses (AdVs) have been intensely studied over several decades, initially due primarily to their roles as human pathogens, but more recently with an additional focus on their prospective use as tools in gene therapy. Research originally focused on a handful of serotypes, but advances in sequencing technology have led to the discovery of close to 100 different human AdV types (HAdVs) [1–4] [http://hadvwg.gmu.edu]. Humans, however, are not the only species infected by AdVs. Members of the diverse family adenoviridae have been shown to infect a wide variety of vertebrate species, including rodents and bats. AdVs are generally considered host-specific viruses, with a few exceptions including CAdV-1 (canine), which infects a broad set of carnivores, and some SAdVs (simian), where transmission between primates and humans has been documented or suspected [1, 5–10].

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Abbreviations: AdV, adenovirus; CAdV, canine adenovirus; HAdV, human adenovirus; USAID, United States Agency for International Development.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Ten supplementary files are available from Figshare.

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Taking the general host specificity of AdVs into account, each host species should have its own set of AdVs, though for most species other than humans we only know of very few, one or no AdVs per host. Considering the high diversity of HAdVs, it seems likely that there may be many AdV types in each of many host species which have remained undetected so far. Identifying and studying these viruses can provide insights into what drives AdV evolution, mechanisms of pathogenicity, and general virus and host biology. The most promising hosts in which to look for AdVs in terms of characterizing evolution are certainly rodents and bats, as these two orders alone account for over 70% of all living mammal species, and recent studies have indeed unveiled many previously unknown AdVs in these hosts [2, 11–16]. Bats in particular have proven to be a rich source of viral discoveries in recent years and have been the subject of many studies evaluating the risks of zoonotic viral transmission from bats to humans or to other hosts. Viruses of significant impact on humans such as Ebola, corona and rabies viruses in bats have been extensively studied but AdVs, while less extensively studied, have also been detected in various species of bats from different continents with prevalences of up to almost 15% [14–23]. Previous studies mostly support a high diversity and the general pattern of virus–host co-evolution in AdVs; however, there is evidence that the latter principle may not be as strict as previously thought [23, 24].

The present study was part of the global United States Agency for International Development (USAID) Emerging Pandemic Threats PREDICT project, aiming at the identification of viral risks at the human–animal interface. The goal of this specific study was to test bats in Cameroon for evidence of current AdV infection to evaluate (i) the AdV (DNA) prevalence in the tested population, (ii) the diversity of AdVs among the bats, (iii) correlations between host species and detected viruses, and (iv) to determine what these can tell us about AdV evolution.

**METHODS**

**Sampling**

Samples from bats were collected between January 2009 and April 2013 in multiple locations in Cameroon (CMR) (Fig. 1, File S3). Bats were obtained from hunters who were willing to let us sample the individuals they captured. Opportunistic samples were taken and hunted bats returned to the hunter after specimen collection. Two specimens of about 200 mg (pea-size) samples from liver and spleen were collected. For each tissue, one specimen was placed in an empty 1.5 ml cryovial and the other in 1.5 ml cryotube containing 500 µl Rnalater (Qiagen). All specimens were frozen immediately in liquid nitrogen in the field and later stored at −80 °C at the laboratory until further processing was carried out.

**PCR and sequence analysis**

DNA was extracted from the specimens using a Qiagen AllPrep DNA/RNA kit, according to the manufacturer's instructions. DNA was stored at −20 °C until analysis. A nested PCR was used to screen for AdV DNA in pooled liver/spleen samples separate for each bat. The selected PCR targets the conserved DNA polymerase gene and amplifies a product of approximately 270–276 nt flanked by the primer binding sites. The primers for the first round (polFouter, 5′-TIM GNG GIG GIM GNT GYT AYC C-3′, and polRouter, 5′-GTD GCR GIG GIM GNT GYT AYC C-3′) and for the second round (polFiner, 5′-GTI TWY GAY ATH TGY GGH ATG TAY GC-3′, and polRInner, 5′-CCA BCD RTT RTG IAR IGT RA-3′) of the PCR are degenerated to enable the detection of a variety of AdVs [25]. To confirm the correct field-identification of bats, DNA samples from representatives of different field-identified species were tested with a cytochrome b (CytB) PCR assay and the products were sequenced. The primers CytB_F (5′-GAG GMC AAA TAT CAT TCT GAG G-3′) and CytB_R (5′-TAG GGC VAG GAC TCC TCC TAG T-3′) were used to amplify a primer-flanked 435 nt fragment of the highly conserved mitochondrial gene [26].

For visualization, all PCR products were loaded into and run in a 1.5% agarose gel, and products corresponding to the expected size were excised. DNA was extracted using either the Qiagen QIAquick gel extraction kit or the MP Biomedicals GeneClean kit and were sent for commercial Sanger sequencing at GATC Biotech. Sequencing results were assessed and processed using Geneious 7.1, and compared to the GenBank database (blastn).

Two phylogenetic trees were reconstructed for viral sequences, one based on the PCR target region in the polymerase gene, and one based on available sequences of the full polymerase gene. For the host species, full or close to full (>1029 nt) CytB sequences were used for phylogenetic analysis. Published bat AdV DNA sequences covering the PCR target region (n=178) were included in the PCR-based tree, provided they did not match other published sequences by more than 90%, in which case only one (the first in the preliminary multiple sequence alignment) of the putative species was included (File S2). Novel isolates with more than 90% nucleotide identities to
other new isolates were also omitted during reconstruction, but are marked in the tree next to included ones. The same 90% nucleotide identity cut-off was used as an inclusion criterion for the full polymerase gene tree. The host CytB sequences selected represent AdV hosts reported either previously or in this study.

For the purpose of phylogenetic analysis, multiple sequence alignments were made in Geneious (version 11.1.3, muscle alignment) (Files S4–S6). Bayesian phylogenies were inferred using MrBayes 3.2 [27]: datatype=DNA, Nucmodel=4by4, Nst=1, Coavion=no, no. states=4, rates=equal, two runs; four chains of 50000000 generations for virus PCR target regions, 10000000 generations for host gene sequences and 1000000 for virus full polymerase gene sequences. Fowl aviadenovirus 1 and 2, as members of the genus Aviadenovirus, served as the outgroup to root the PCR trees, Gallus gallus and Anas platyrhynchos were used as the outgroup for CytB trees, and Murid adenovirus 1 and 3 as the outgroup for virus full polymerase trees. Trees were sampled after every 1000 steps during the process to monitor phylogenetic convergence. The mean standard deviation of split frequencies was below 0.0184 for the AdV PCR target region analysis, below 0.0081 for the CytB analysis and 0 for the virus full polymerase analysis. The first 10% of the trees were discarded and the remaining ones combined using TreeAnnotator (versions 1.10.4 and 2.5.1; http://beast.bio.ed.ac.uk) and displayed with FigTree (1.4.4; http://tree.bio.ed.ac.uk/). Sequences obtained from the CytB PCR were ‘blasted’ against the GenBank database and were considered to match a species if identities were at 98% or higher.

**Statistical analysis**

Ecological data collected for each animal was analysed with respect to a correlation with the frequency of virus detection. We assessed the effect of sex (male vs female), season the sample was taken (rainy – May to September, dry – October to April) and colony behaviour on the likelihood of AdV DNA being detected in an individual. Colony behaviour
was coded as an ordinal variable, specifically categorizing species as solitary (code=1), mixed solitary and small colonies (code=2), small colonies (code=3), mixed small and large colonies (code=4), and bats that exhibit highly colony-based/gregarious behaviour based on published references and the IUCN (International Union for Conservation of Nature) Red List (Table 1) [28–30]. The likelihood of detecting AdV DNA in an individual animal based on these variables was assessed by fitting a multivariate general linear binomial model with a logit link (R v. 3.4.0). There were insufficient numbers of individuals in many species to consider species as a predictor, and so inferences are limited to the taxa 'bat'. We did, however, have sufficient samples of bats to compare the likelihood of detecting AdV DNA from the families Hipposideridae and Pteropodidae using a univariate analysis (Chi-square contingency table). There were insufficient numbers of bats from other families (i.e. n <35 for the remaining six families represented) to include family as a covariate in the model.

For cospeciation analysis, the Jane software tool (version 4) with default settings for generations and population size was used with the host and virus trees (Figs 2 and 3) [31]. In order to run the software virus sequences without the corresponding host, CytB was excluded as well as bat AdV species 15, 18 and 35. Default costs for events were used (cospeciation 0, duplications 1, duplications and host switches 2, losses 1, failure to diverge 1) and more extreme costs (10) for each category explored.

Correlations between the AdV phylogeny of the PCR-based tree and the geographical location of sample collection were evaluated using the BaTS software package [32]. Locations ('states') were either coded as the country or as the continent from which a sequence originated.

**RESULTS**

**Roosting behaviour correlates with AdV prevalence**

During the course of this study, 671 bats from 37 different species were sampled in various regions and habitats in Cameroon (Fig. 1, File S3). The most commonly sampled species were *Epomops franqueti* (124, 18.5%), *Hipposideros ruber* (108, 16.1%) and *Megaloglossus woermanni* (108, 16.1%), while other species were represented by fewer or only a single individual (Table 1). AdV DNA was detected in 94 (14%) of the bats, with detection rates ranging from 0 to 100% for various species. We were more likely to detect AdV DNA in samples from bats of the family Hipposideridae than the family Pteropodidae (Chi-square=14.34, df=1, P<0.001; Hipposideridae 21.7% positive, n=161; Pteropodidae 9.5% positive, n=411). We did not detect an effect of season (12.6% positive in the dry season vs 16.7% in the rainy season; β=0.34, SE=0.24, P=0.15, n=671) or sex (12.8% positive among females vs 15.6% positive among males; β=0.27, SE=0.23, P=0.24, n=666; 5 individuals with undetermined sex were excluded) (File S7). We did detect a correlation between degree of colony behaviour and proportion of individuals testing positive for AdV (β=0.28, SE=0.09, P=0.00145, n=654; **Table 1.** Sampled bat species

| Species | No. AdV positive/total no. sampled (% AdV positives) | Roosting behaviour |
|---------|-----------------------------------------------------|-------------------|
| **Pteropodidae** | | |
| Casiemycteris argyrmis | 0/2 (0%) | Solitary |
| Eidolon helvum | 1/1 (100%) | Large colonies |
| Epomophorus gambianus | 0/9 (0%) | Large colonies |
| Epomops franqueti | 5/124 (4%) | Small colonies |
| Hypsignathus monstrosus | 1/4 (25%) | Small colonies |
| Lissonycteris angolensis | 2/19 (10.5%) | Small colonies |
| Megaloglossus woermanni | 14/108 (13%) | Solitary |
| Micropteropus pusillus | 1/68 (1.5%) | Solitary |
| Myonycteris torquata | 0/25 (0%) | Small colonies/solitary |
| Myonycteris sp. | 2/2 (100%) | Unknown |
| Nanonycteris veldkampi | 0/3 (0%) | Unknown |
| Rousettus aegyptiacus | 15/43 (34.9%) | Large colonies |
| Scotonycteris zenkeri | 0/5 (0%) | Small colonies |
| **Hipposideridae** | | |
| Hipposideros beatus | 0/2 (0%) | Small colonies/solitary |
| Hipposideros cyclops | 15/20 (75%) | Small colonies |
| Hipposideros fuliginosus | 0/3 (0%) | Small colonies |
| Hipposideros gigas | 3/28 (10.7%) | Large colonies |
| Hipposideros ruber | 17/108 (15.7%) | Large colonies/small colonies |
| **Rhinolophidae** | | |
| Rhinolophus alicyone | 4/5 (80%) | Small colonies/solitary |
| Rhinolophus fumigatus | 1/1 (100%) | Large colonies |
| Rhinolophus landeri | 0/8 (0%) | Small colonies |
| **Rhinopomatidae** | | |
| Rhinopoma microphyllum | 0/1 (0%) | Large colonies/small colonies |
| **Molossidae** | | |
| Chaerephon pumilus | 2/3 (66.7%) | Large colonies |
| Mops condylurus | 3/25 (12%) | Large colonies/small colonies |
| Mops demonstrator | 0/6 (0%) | Small colonies |
| **Vespertilionidae** | | |
| Eptesicus capensis | 0/2 (0%) | Small colonies |

Continued
17 animals from species with unknown roosting behaviour were excluded) (Fig. 4).

**Detected AdV DNA belongs to different AdV species**

The majority of the sequences obtained from the 94 AdV DNA positive samples did not resemble previously reported sequences. Only 22 of the isolates had GenBank matches with nucleotide identities of 85% or more, and only 2 of these (CMR-5952 and CMR-5697) were identical with known bat AdV sequences (Table 2). With a cut-off of 5–15% differences in the amino acid sequence, the 94 virus isolates could be classified into 28 to 44 different mastadenovirus species [2]. Upon phylogenetic analysis, novel isolates were found in all major branches of the tree alongside other AdV isolates from a diverse set of bat host species sampled in different locations in Africa, Asia and Europe, while some of the novel isolates formed exclusive clusters (Fig. 2). Phylogeny-trait association tests based on monophyletic clade (MC) size statistic of virus-location phylogenetic substructure do not reject the null hypothesis of no association between virus and location, as MC for all states (countries and continents) is >0.01.

**Highly social bats seem to be infected by more AdV species**

The proportion of samples with a unique AdV species (number of different AdV species detected/number of samples collected) differed depending on the behaviour category. Bats roosting in large colonies carried a significantly more diverse set of AdVs compared to all other categories combined (Chi-square=13.32, df=1, *P* < 0.001; 'large colonies' 15.1%, *n*=86; 'all other' 4.9%, *n*=571) or individually, while in solitary bats the proportion was the lowest (Chi-square=5, df=1, *P* < 0.026; 'solitary' 2.8%, *n*=178; 'all other' 7.5%, *n*=478).

In the 7 bat species that were roosting in large colonies and were sampled, a total of 13 distinct AdVs were detected (ratio of 1.9 AdVs per species), while in the 26 species in all the other categories combined, 28 distinct AdVs were detected (ratio of 1.1 AdVs per species). A similar trend for more AdV species among large colony roosting bats can be seen in the GenBank data set, with 11 AdV species deriving from 5 bat species (ratio of 2.2 AdVs per species) compared to 68 AdVs derived from 44 bat species across all other categories (ratio of 1.5 AdVs per species) (Files S2 and S8).

**Virus and host phylogenies differ**

A CytB-based phylogenetic analysis of all bat species from which AdV DNA was isolated and reported produced a tree consistent with current bat taxonomy (Fig. 3). The host and the PCR-fragment-based virus trees do not mirror each other, but branches in the virus tree are largely bat *suborder* specific and contain either viruses of *Yinpterochiroptera* or *Yangochiroptera* hosts. A virus tree calculated based on full genome sequences does not provide the same level of resolution due to the limited number of available sequences, but with high bootstrap supports indicates that the split of *Yinpterochiroptera* and *Yangochiroptera* bats is not well reflected in the infecting AdV's phylogeny (File S9). Most of the larger clusters in the PCR-fragment-based virus tree represent only one family of bats, but some families, such as *Vespertilionidae* and *Pteropodidae*, are represented with more than one of these exclusive clusters (Fig. 2). A consistent clustering down to the host genus level is not visible, but 75% (15/20) of the virus species detected in more than one animal were only found in one species (Table 2). Cospeciation analysis with Jane indicates diverse modes as drivers for the calculated phylogeny with cospeciation (9), duplication of parasites (27), duplication and host switch (52), loss of parasite (105), failure to diverge (18), at a total cost of 254.

**DISCUSSION**

We detected AdV DNA in 14% of the tested bats, which is close to the upper end of what had been reported previously and, thus, very similar to what Vidovsky *et al.* found in bats in Europe [14]. However, such an overall prevalence has to be considered with caution, as many ecological factors may have an influence on this. Like Vidovsky *et al.*, we found a large variation in prevalence between the bat species (0–100%), and the species composition of tested bat populations is probably one of the main factors contributing to the prevalence differences among studies [14–23]. The 37 bat species we sampled were not equally represented, with over a hundred individuals tested from each of three species and only a single or few individuals of some other species (Table 1). Therefore, the prevalences we observed in some species may not necessarily be representative. However, the 671 bats tested do
Fig. 2. Virus phylogeny: phylogenetic tree of bat AdVs based on the PCR target region (270–276 nt) of the PCR described by Wellehan et al. [25]. Previously published sequences are indicated by GenBank accession number, host species and sampling location, novel isolates (in green boxes) by species numbers and the hosts. Bootstrap support is shown on nodes if higher than 0.5. The branches of AdVs from *Yinpterochiroptera* bats are coloured in red, branches of AdVs from *Yangochiroptera* bats in blue, with purple indicating branches with viral isolates that were found in both. Branches with funnel like ends indicate that one sequence is representing several new isolates.
Fig. 3. Phylogenetic tree of bats: phylogenetic tree of bat species based on the full or near full cytochrome B gene. Bootstrap support is shown on knots. The branches of Yinpterochiroptera bats are coloured in red, branches of Yangochiroptera bats in blue. The sequences included are: AB355764 (Eidolon helvum), AB085740 (Rousettus aegyptiacus), FJ549336 (Rousettus leschenaultii), JN398205 (Lissonycteris angolensis), JF728759 (Myonycteris torquata), JF728758 (Epomops franqueti), KT875803 (Epomophorus gambianus), JN398208 (Micropteropus pusillus), KX823086 (Hypsipyla monstrosa), DQ445710 (Megaloglossus woermanni), AB042770 (Pteropus dasymallus), MH686226 (Cardiodera cor), EU436941 (Rhinolophus sinicus), DQ351848 (Rhinolophus ferrumequinum), FJ457614 (Rhinolophus fumigatus), EU436671 (Rhinolophus euryale), FJ457613 (Rhinolophus alcyone), EU934469 (Hipposideros giga), EU934466 (Hipposideros cyclops), JX849190 (Hipposideros armiger), FJ437988 (Hipposideros rufus), FJ437980 (Hipposideros caffer), EU750931 (Scotophilus kuhlii), GQ272582 (Eptesicus nilssonii), EU751000 (Eptesicus serotinus), KF094115 (Pipistrellus pipistrellus), AJ504442 (Pipistrellus pygmaeus), KU058655 (Pipistrellus kuhlii), CQ332430 (Pipistrellus abramus), KX570901 (Nyctalus leisleri), XJ570900 (Nyctalus lasiopterus), AJ504450 (Hypsugo savii), KJ756000 (Neoromicia capensis), LC052293 (Vespertilio murinus), AB287362 (Vespertilio sinensis), EF570882 (Plecotus auritus), AY776085 (Corynorhinus rafinesqui), KP187907 (Myotis horsfieldii), KX467599 (Myotis ricketti), EF555226 (Myotis emarginatus), AB085736 (Myotis macrodactylus), GUS17388 (Myotis myotis), KX467686 (Myotis blythii), KX467688 (Myotis macrotis), AF376849 (Myotis emarginatus), AF376846 (Myotis dasycneme), MF615184 (Myotis ricketti), EF555226 (Myotis imbricatus), AB085736 (Myotis macrodactylus), GUS17388 (Myotis myotis), KX467686 (Myotis blythii), KX467688 (Myotis macrotis), AF376849 (Myotis emarginatus), AF376846 (Myotis dasycneme), MF615184 (la io), AY591536 (Otoms martensi), MK330941 (Mops condylurus), GQ489157 (Charephon pumilus), HQ693723 (Nycteris hispida), KX548053 (Miniopterus natalensis), FJ232806 (Miniopterus minor), AY208140 (Miniopterus schreibersii), JQ956449 (Coleura atra), EU755252 (Anas platyrhynchos) and AB04486 (Gallus gallus).
Fig. 4. Roosting behaviour correlates with AdV prevalence: proportion of virus detections in animals in relation to roosting behaviour. Total animals sampled per category and margin of error for 95% confidence intervals are shown.

Fig. 4. Roosting behaviour correlates with AdV prevalence: proportion of virus detections in animals in relation to roosting behaviour. Total animals sampled per category and margin of error for 95% confidence intervals are shown.

represent a substantial range of species with different reported roosting behaviours, and the data suggests a correlation between bats dwelling in large caves or in big groups and bats having a higher prevalence of AdV infections (Fig. 4). This observation also aligns with what is known generally about AdV transmission, which has been shown to be more likely to be increased under crowded conditions, an example being HAdV outbreaks in army camps [1].

The diversity of the detected AdVs was high since, according to the International Committee on Taxonomy of Viruses (ICTV) criteria, 28–44 of the 94 detected sequences might represent unique AdV species, depending on where the line is drawn [2]. Based on the comparison of the amino acid sequences of the full gene and the PCR target region of 16 common mastadenoviruses, we conclude that the amplified region is generally more conserved than the whole gene; hence, the number of viral species may be closer to the higher end; for the tree we were assuming 38. Sequencing of the whole gene or full genomes would have allowed for a clearer classification; however, since for most other bat AdVs only the PCR region has been sequenced, it would not have had much influence on the analysis. Considering that the assumed 28–44 AdV species were detected in only 17 different bat species, we are looking at a ratio of between 1.6 and 2.6 AdV species per host species. This is higher than what has been observed, for example, in rodents, and similar to what was found in bats elsewhere [14–16]. This supports the hypothesis that the diverse order Chiroptera might still harbour hundreds of yet unknown AdVs [14].

Virus–host co-evolution and species specificity have generally been regarded as the driving factors for AdV evolution and diversity, despite the relative host ambiguity of CAdV-1 [5, 7, 24]. However, recent findings, such as evidence for human–primate zoonotic events and results from surveys in rodents and bats, indicate that matters may be more complex [1, 6, 8–12, 14–16, 23]. The results of the Jane analysis, with relatively few (9) predicted co-speciation events and an overall high total cost of 254 (the higher the influence of co-speciation the closer it would be to 0), also point towards a complex evolution of bat AdVs that involves, but not primarily depends on, co-evolution. As a consequence of the majority of sequences obtained by us and others being relatively short, bootstrap support for many nodes in the phylogenetic tree is not optimal, so that the location of various nodes and branches has to be viewed with some caution (Fig. 2). Our analysis of a large number of bat AdV sequences, nevertheless, suggests that species specificity and co-evolution alone may not be sufficient to explain the observed diversity. The
| Species | Isolate/GenBank acc. no. | Host family and species | BLAST N (29/05/18) | Location* |
|---------|-------------------------|-------------------------|-------------------|-----------|
| 1       | CMR-5440, MN136559      | Pteropodidae, Epomops franqueti | 80% unidentified adenovirus isolate PgAdV-1 (KC692417) | ES        |
|         | CMR-5586, MN136577      | Pteropodidae, Epomops franqueti | 80% unidentified adenovirus isolate PgAdV-1 (KC692417) | SU        |
|         | CMR-5628, MN136584      | Pteropodidae, Epomops franqueti | 81% unidentified adenovirus isolate PgAdV-1 (KC692417) | SU        |
| 2       | CMR-5524, MN136570      | Pteropodidae, Megaloglossus woormanni | 77% unidentified adenovirus isolate PgAdV-1 (KC692417) | ES        |
|         | CMR-5529, MN136573      | Pteropodidae, Megaloglossus woormanni | 76% unidentified adenovirus isolate PgAdV-1 (KC692417) | ES        |
|         | CMR-5531, MN136574      | Pteropodidae, Epomops franqueti | 77% unidentified adenovirus isolate PgAdV-1 (KC692417) | ES        |
|         | CMR-5646, MN136586      | Pteropodidae, Megaloglossus woormanni | 77% unidentified adenovirus isolate PgAdV-1 (KC692417) | SU        |
| 3       | CMR-5587, MN136578      | Pteropodidae, Rousettus aegyptiacus | 99% unidentified adenovirus isolate CS/13HN77 (KT369241) | SU        |
| 4       | CMR-5459, MN136560      | Vespertilionidae, Pipistrellus musciculus | 77% mastadenovirus sp. strain BatAdVNeoV17-2 (MF593274) | SU        |
|         | CMR-5470, MN136561      | Vespertilionidae, Epomops franqueti | 76% vespertilionid adenovirus 4 strain 492/08 (KM043107) | SU        |
|         | CMR-5481, MN136563      | Nycteridae, Nycteris grandis | 77% mastadenovirus sp. strain BatAdVNeoV17-2 (MF593274) | SU        |
| 5       | CMR-5238, MN136544      | Vespertilionidae, Neoromicia tenaiipinnis | 82% mastadenovirus sp. strain BatAdVNeoC52222 (MF593281) | LI        |
|         | CMR-5241, MN136545      | Vespertilionidae, Neoromicia tenaiipinnis | 81% mastadenovirus sp. strain BatAdVNeoC52222 (MF593281) | LI        |
|         | CMR-5242, MN136546      | Vespertilionidae, Neoromicia tenaiipinnis | 82% mastadenovirus sp. strain BatAdVNeoC52222 (MF593281) | LI        |
| 6       | CMR-5263, MN136547      | Rhinolophidae, Rhinolophus alycune | 74% bat mastadenovirus WIV10 (KT698854) | LI        |
|         | CMR-5679, MN136588      | Rhinolophidae, Rhinolophus alycune | 75% bat mastadenovirus WIV11 (KT698855) | SW        |
|         | CMR-5984, MN136626      | Pteropodidae, Rousettus aegyptiacus | 77% bat mastadenovirus WIV10 (KT698854) | SW        |
|         | CMR-6223, MN136629      | Hipposideridae, Hipposideros cyclops | 75% bat mastadenovirus WIV11 (KT698855) | CE        |
| 7       | CMR-5201, MN136540      | Hipposideridae, Hipposideros ruber | 71% unidentified adenovirus isolate Aaa12186 (MF175106) | ES        |
|         | CMR-5713, MN136598      | Hipposideridae, Hipposideros ruber | 72% bat adenovirus isolate 1391-YN-Ha (GU226964) | ES        |
|         | CMR-5716, MN136600      | Hipposideridae, Hipposideros ruber | 71% canine mastadenovirus A isolate CAdV-1 ITL2015 (KX545420) | ES        |
| 8       | CMR-5232, MN136543      | Molossidae, Chaerephon pumilus | 73% bat adenovirus strain C018/China/2015 (KY009658) | LI        |
|         | CMR-5929, MN136618      | Molossidae, Chaerephon pumilus | 74% bat adenovirus strain C018/China/2015 (KY009658) | N         |
| Species | Isolate/GenBank acc. no. | Host family and species | BLAST n (29/05/18) | Location* |
|---------|--------------------------|-------------------------|---------------------|-----------|
| 9       | CMR-5379, MN136549       | Hipposideridae, Hipposideros cyclops | 74% unidentified adenovirus isolate Aau12186 (MF175106) | ES        |
|         | CMR-5381, MN136550       | Hipposideridae, Hipposideros cyclops | 73% unidentified adenovirus isolate Aau12186 (MF175106) | ES        |
|         | CMR-5382, MN136551       | Hipposideridae, Hipposideros cyclops | 73% unidentified adenovirus isolate Aau12186 (MF175106) | ES        |
|         | CMR-5395, MN136553       | Hipposideridae, Hipposideros cyclops | 73% unidentified adenovirus isolate Aau12186 (MF175106) | ES        |
|         | CMR-5397, MN136555       | Hipposideridae, Hipposideros cyclops | 73% unidentified adenovirus isolate Aau12186 (MF175106) | ES        |
|         | CMR-5398, MN136556       | Hipposideridae, Hipposideros cyclops | 73% unidentified adenovirus isolate Aau12186 (MF175106) | ES        |
|         | CMR-5634, MN136585       | Hipposideridae, Megaloglossus woermanni | 73% unidentified adenovirus isolate Aau12186 (MF175106) | ES        |
|         | CMR-5793, MN136607       | Hipposideridae, Hipposideros cyclops | 72% unidentified adenovirus isolate Aau12186 (MF175106) | SU        |
|         | CMR-5998, MN136627       | Hipposideridae, Hipposideros cyclops | 74% unidentified adenovirus isolate Aau12186 (MF175106) | LI        |
|         | CMR-6211, MN136628       | Hipposideridae, Myonycteris sp. | 75% unidentified adenovirus isolate Aau12186 (MF175106) | CE        |
| 10      | CMR-5378, MN136548       | Hipposideridae, Hipposideros cyclops | 72% unidentified adenovirus isolate Aau12186 (MF175106) | ES        |
|         | CMR-5795, MN136609       | Hipposideridae, Hipposideros cyclops | 74% unidentified adenovirus isolate Aau12186 (MF175106) | SU        |
| 11      | CMR-5394, MN136552       | Hipposideridae, Hipposideros cyclops | 70% unidentified adenovirus isolate Aau12186 (MF175106) | ES        |
| 12      | CMR-5416, MN136558       | Hipposideridae, Hipposideros gigas | 71% Mediterranean horseshoe bat adenovirus 3 strain BS12 (KM043081) | SU        |
|         | CMR-5414, MN136557       | Hipposideridae, Hipposideros gigas | 80% unidentified adenovirus isolate Aau12186 (MF175106) | SU        |
|         | CMR-5862, MN136610       | Hipposideridae, Hipposideros gigas | 73% unidentified adenovirus isolate Aau12186 (MF175106) | SU        |
| 13      | CMR-5603, MN136582       | Hipposideridae, Megaloglossus woermanni | 81% Mediterranean horseshoe bat adenovirus 2 strain BS12 (KM043080) | SU        |
| 14      | CMR-5206, MN136541       | Hipposideridae, Hipposideros ruber | 81% Kuhl's pipistrelle adenovirus 1 strain 09/09 (KM043100) | ES        |
|         | CMR-5213, MN136542       | Hipposideridae, Hipposideros ruber | 81% Kuhl's pipistrelle adenovirus 1 strain 09/09 (KM043100) | ES        |
|         | CMR-5499, MN136565       | Hipposideridae, Hipposideros ruber | 81% Kuhl's pipistrelle adenovirus 1 strain 09/09 (KM043100) | SU        |
|         | CMR-5901, MN136616       | Hipposideridae, Hipposideros ruber | 82% Kuhl's pipistrelle adenovirus 1 strain 09/09 (KM043100) | SW        |
| 15      | CMR-5508, MN136567       | Hipposideridae, Hipposideros ruber | 73% marten adenovirus type 2 isolate PM04 (KY753134) | SU        |
|         | CMR-5509, MN136568       | Hipposideridae, Hipposideros ruber | 73% marten adenovirus type 2 isolate PM04 (KY753134) | SU        |
|         | CMR-5510, MN136569       | Hipposideridae, Hipposideros ruber | 73% marten adenovirus type 2 isolate PM04 (KY753134) | SU        |
| Species | Isolate/GenBank acc. no. | Host family and species | BLAST N (29/05/18) | Location* |
|---------|-------------------------|-------------------------|-------------------|-----------|
| 17      | CMR-5899, MN136614      | Hipposideridae, Hipposideros ruber | 74% marten adenovirus type 2 isolate PM04 (KY753134) | SW        |
|         | CMR-5900, MN136615      | Hipposideridae, Hipposideros ruber | 74% marten adenovirus type 2 isolate PM04 (KY753134) | SW        |
|         | CMR-5965, MN136622      | Hipposideridae, Hipposideros ruber | 74% marten adenovirus type 2 isolate PM04 (KY753134) | SW        |
|         | CMR-5968, MN136623      | Hipposideridae, Hipposideros ruber | 74% marten adenovirus type 2 isolate PM04 (KY753134) | SW        |
| 18      | CMR-5504, MN136566      | Hipposideridae, Hipposideros ruber | 75% marten adenovirus type 2 isolate PM04 (KY753134) | SU        |
|         | CMR-5715, MN136599      | Hipposideridae, Hipposideros ruber | 73% marten adenovirus type 2 isolate PM04 (KY753134) | ES        |
|         | CMR-5974, MN136624      | Hipposideridae, Hipposideros ruber | 72% marten adenovirus type 2 isolate PM04 (KY753134) | SW        |
| 19      | CMR-5691, MN136593      | Pteropodidae, Rousettus aegyptiacus | 98% bat adenovirus isolate BAT0025/Kwale (KY311889) | SW        |
| 20      | CMR-5690, MN136592      | Pteropodidae, Rousettus aegyptiacus | 79% unidentified adenovirus isolate PgAdV-10 (KC692426) | SW        |
| 21      | CMR-5704, MN136597      | Pteropodidae, Rousettus aegyptiacus | 79% unidentified adenovirus isolate PgAdV-9 (KC692425) | SW        |
| 22      | CMR-5735, MN136601      | Vespertilionidae, Neoromicia tenispinnis | 76% brown long-eared bat adenovirus 1 strain 345/08 (KM043094) | LI        |
| 23      | CMR-5758, MN136603      | Nycteridae, Nycteris hispida | 76% vespertilionid adenovirus 4 strain 492/08 (KM043107) | SU        |
| 24      | CMR-5868, MN136611      | Molosidae, Mops condylurus | 73% bottlenose dolphin adenovirus 1 strain Ti11018 (KR024710) | NW        |
|         | CMR-5871, MN136612      | Molosidae, Mops condylurus | 73% bottlenose dolphin adenovirus 1 strain Ti11018 (KR024710) | NW        |
| 25      | CMR-5877, MN136613      | Molosidae, Mops condylurus | 75% Mediterranean horseshoe bat adenovirus 3 strain BS12 (KM043081) | NW        |
| 26      | CMR-5928, MN136617      | Rhinolophidae, Rhinolophus fumigatus | 84% Mediterranean horseshoe bat adenovirus 3 strain BS12 (KM043081) | N         |
| 27      | CMR-5703, MN136596      | Pteropodidae, Rousettus aegyptiacus | 79% unidentified adenovirus isolate PgAdV-9 (KC692425) | SW        |
| 28      | CMR-5736, MN136602      | Vespertilionidae, Neoromicia tenispinnis | 74% phocine adenovirus 1 isolate PhAdV-1 (JX244191) | LI        |
| 29      | CMR-5952, MN136619      | Pteropodidae, Euderma helvum | 100% bat adenovirus isolate BAT0066/Kisii (KY311885) | SW        |
| 30      | CMR-6226, MN136630      | Rhinolophidae, Rhinolophus alyceone | 75% unidentified adenovirus isolate Aau13092 (MF175107) | CE        |
|         | CMR-6227, MN136631      | Rhinolophidae, Rhinolophus alyceone | 74% bottlenose dolphin adenovirus 1 strain Ti11018 (KR024710) | CE        |
| 31      | CMR-5491, MN136564      | Pteropodidae, Rousettus aegyptiacus | 80% unidentified adenovirus isolate PgAdV-10 (KC692426) | SU        |
| 32      | CMR-5396, MN136554      | Hipposideridae, Hipposideros cyclops | 71% unidentified adenovirus isolate Aau12186 (MF175106) | ES        |
| 33      | CMR-5792, MN136606      | Hipposideridae, Hipposideros cyclops | 73% Mediterranean horseshoe bat adenovirus 3 strain BS12 (KM043081) | SU        |
| 34      | CMR-5794, MN136608      | Hipposideridae, Hipposideros cyclops | 71% unidentified adenovirus isolate Aau12186 (MF175106) | SU        |
| 35      | CMR-5687, MN136590      | Pteropodidae, Rousettus aegyptiacus | 80% unidentified adenovirus isolate PgAdV-9 (KC692425) | SW        |
| 36      | CMR-6243, MN136632      | Pteropodidae, Micropterus pusillus | 79% unidentified adenovirus isolate PgAdV-11 (KC692427) | N         |
| Species | Isolate/GenBank acc. no. | Host family and species | Blast n (29/05/18) | Location* |
|---------|-------------------------|-------------------------|--------------------|-----------|
| 35      | CMR-5983, MN136625      | Pteropodidae, Rousettus aegyptiacus | 76% unidentified adenovirus isolate PgAdV-11 (KC692427) | SW        |
| 36      | CMR-5471, MN136562      | Pteropodidae, Megaloglossus woermanni | 89% bat mastadenovirus WIV17 (KX961095) | SU        |
|         | CMR-5525, MN136571      | Pteropodidae, Megaloglossus woermanni | 89% bat mastadenovirus WIV17 (KX961095) | ES        |
|         | CMR-5528, MN136572      | Pteropodidae, Megaloglossus woermanni | 90% bat mastadenovirus WIV17 (KX961095) | ES        |
|         | CMR-5563, MN136575      | Pteropodidae, Hypsignathus monstrosus | 89% bat mastadenovirus WIV18 (KX961096) | SU        |
|         | CMR-5566, MN136576      | Pteropodidae, Megaloglossus woermanni | 89% bat mastadenovirus WIV17 (KX961095) | SU        |
|         | CMR-5591, MN136579      | Pteropodidae, Megaloglossus woermanni | 90% bat mastadenovirus WIV17 (KX961095) | SU        |
|         | CMR-5594, MN136580      | Pteropodidae, Megaloglossus woermanni | 90% bat mastadenovirus WIV17 (KX961095) | SU        |
|         | CMR-5600, MN136581      | Pteropodidae, Megaloglossus woermanni | 90% bat mastadenovirus WIV17 (KX961095) | SU        |
|         | CMR-5609, MN136583      | Pteropodidae, Megaloglossus woermanni | 90% bat mastadenovirus WIV17 (KX961095) | SU        |
|         | CMR-5953, MN136620      | Pteropodidae, Megaloglossus woermanni | 90% bat mastadenovirus WIV17 (KX961095) | SW        |
|         | CMR-5958, MN136621      | Pteropodidae, Lissonycteris angolensis | 89% bat mastadenovirus WIV17 (KX961095) | SW        |
|         | CMR-6265, MN136633      | Pteropodidae, Myonycteris sp. | 91% bat mastadenovirus WIV17 (KX961095) | AD        |
| 37      | CMR-5661, MN136587      | Pteropodidae, Rousettus aegyptiacus | 89% bat mastadenovirus WIV17 (KX961095) | SU        |
|         | CMR-5684, MN136589      | Pteropodidae, Rousettus aegyptiacus | 89% bat mastadenovirus WIV17 (KX961095) | SW        |
|         | CMR-5695, MN136594      | Pteropodidae, Rousettus aegyptiacus | 89% bat mastadenovirus WIV17 (KX961095) | SW        |
|         | CMR-5788, MN136604      | Pteropodidae, Rousettus aegyptiacus | 90% bat mastadenovirus WIV17 (KX961095) | SU        |
|         | CMR-5789, MN136605      | Pteropodidae, Rousettus aegyptiacus | 90% bat mastadenovirus WIV17 (KX961095) | SU        |
| 38      | CMR-5688, MN136591      | Pteropodidae, Rousettus aegyptiacus | 99% bat adenovirus isolate 1 050 597 (HQ529709) | SW        |
|         | CMR-5697, MN136595      | Pteropodidae, Rousettus aegyptiacus | 100% bat adenovirus isolate 1 050 597 (HQ529709) | SW        |

*Administrative regions in Cameroon: Adamawa (AD), Central (CE), East (ES), Littoral (LI), North (N), North-West (NW), South (SU), South-West (SW).
analysis of 10 full gene bat AdV sequences points in the same direction (File S9). The clusters of virus isolates from hosts of the same family and the relatively consistent separation of clusters of viruses from *Yinpterochiroptera* and *Yangochiroptera* supports the established idea that co-evolution plays an important role. A surprising finding was the composition of several clusters with AdVs isolated from hosts belonging to numerous different genera, and presumed virus species infecting various hosts such as species 2, 4, 6, 9 and 36. Thus, the concept of co-evolution does not follow through to the host genus or species level (Table 2, Fig. 2). We see two possible explanations for this observation. In scenario one, the common ancestor of all bats had a large set of AdVs that was passed on to the diverging species, potentially evolving at a slower rate than the host. In scenario two, the common ancestor of all bats had a rather small set of AdVs that was passed on and diverged with the species, while AdVs were and are to some extent exchanged between bat species (File S10). These are not mutually exclusive, but based on the current data, with detections of some very closely related AdVs in different host species, we consider scenario two more likely to play the major role.

Notably, in GenBank we also identified three AdV isolates where the reported host falls into a different suborder than the hosts of related isolates, KY311894 (*Eidolon helvum*), KY311901 (*Otomops martiensseni*) and KY311899 (*Coleura atra*). These three species all form large roosts in trees, caves or ceilings, often in hundreds to tens of thousands of individuals [29]. Those conditions would certainly be ideal to maintain an AdV infection that has successfully jumped a species barrier. Additionally, some of these species occur in caves where multiple species may be present in groups that may be mixed or adjacent. These conditions could potentially lead to a higher diversity of AdVs than in bats that roost in smaller numbers and our data seems to support this. The significantly higher rate of unique AdV species detections among bats roosting in large colonies and the low rate in solitary bats do point in this direction, and so do the observed trends based on the dataset collected for this study and the GenBank dataset that suggest more AdV species per bat species for those roosting in large colonies (File S8). These results have to be interpreted with some caution, since the 37 species we sampled only represent a tiny fraction of Chiroptera diversity, and there likely is some degree of sampling error. Nevertheless, the trend that bat species with preferences for larger roost sizes appear to host a higher diversity of AdVs seems worth following up on. This observation is also consistent with studies finding that larger roost sizes lead to increased transmission of pathogens both in models and with henipaviruses in bats [33, 34].

In summary, this study contributes significantly to our knowledge about the diversity of AdVs harboured by bats, with up to 44 novel AdV species detected. The results of the analysis give us new insights into the evolution of bat AdVs and AdVs in general, as well as the role of their hosts. These findings suggest that host roost size may play an important role in the epidemiology and AdV evolution as it also does for other viruses, and might also be what has limited some AdVs to single species.

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**Author contributions**
Conceptualization, N. D. W. and C. E. L.; methodology, N. D. W. and C. E. L.; validation, M. L., A. G. and C. E. L.; formal analysis, M. L., A. G. and C. E. L.; investigation, N. F. N., J. L. D. D., V. N. N., J.-M. T. and A. G.; resources, J. N.; data curation, M. M. M., M. L., A. G. and C. M.; writing – original draft, J. L., D. D., D. J. M. and C. E. L.; writing – review and editing, all authors; visualization, M. L., D. J. M. and C. E. L.; supervision, U. T., M. M. M., M. L., A. G., B. S. S., C. M., D. O. J., E. M. R. and C. E. L.; project administration, U. T., J. N., C. M., D. O. J. and E. M. R.; funding acquisition, U. T., D. O. J., A. W. R. and N. D. W.

**Conflicts of interest**
The authors declare that there are no conflicts of interest.

**Ethical statement**
Specimen collection was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California Davis, Johns Hopkins University (JHU) (ACR 2007-110-03, 2007-110-02 and 2007-110-11) and University of California Los Angeles (UCLA) (protocols F503MM221 and F506H205), and the Government of Cameroon.

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