Satellite virus diversification through host shifting revealed by novel deltaviruses in vampire bats

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Abstract

Satellite viruses are small, RNA-based hyper-parasites which obligately require ‘helper’ viruses to transmit within and between hosts. The evolutionary pathways through which satellites spread among host species are largely unknown but define their potential as emerging pathogens. Here using metagenomic and field studies of bats, we show that deltaviruses, a medically important group of animal infecting satellites, are capable of transmitting between host species. Among 44 bat genera from 11 countries spanning 5 continents, deltaviruses were exclusively found in a single neotropical sanguivore, the common vampire bat, which harbored two divergent genotypes. Phylogenetic analysis showed that the vampire bat-associated deltaviruses arose independently, implying multiple introductions to bats and the existence of additional, currently undiscovered deltavirus reservoirs in the Americas. Field studies in Peru revealed that deltavirus infections were widespread in vampire bats, formed geographically compartmentalized transmission cycles, and were capable of transmitting to a sympatric bat species. Despite sharing most recent common ancestry with the human pathogen hepatitis deltavirus (HDV), bat deltaviruses were detected without hepadnaviruses (the canonical helper of HDV), implying helper switching during the divergence of bat and human-infecting deltaviruses. While the biological consequences of deltavirus host and helper switching are unknown, it is reasonable to expect they will be aligned with the previously defined ability of satellites to manipulate the virulence and transmissibility of their helpers.

Introduction

Satellite viruses are subviral agents which obligately require unrelated helper viruses to complete their life cycles. A wide variety of plant and animal-infecting satellites modulate the virulence and transmissibility of their helpers, which creates important impacts on agriculture and human health (1). For instance, the human pathogen hepatitis delta virus (HDV), is a circular, single-stranded 1,700 nucleotide (nt) RNA satellite of hepatitis B virus (HBV, family *Hepadnaviridae*) (2), which causes the most severe form of viral hepatitis in ca. 20 million people co-infected with HBV globally (3,4). Although HDV is replicated by diverse non-human cells, dependence on HBV for cellular entry and egress was long thought to limit HDV infections to HBV-infected humans (3,5). Indeed, HDV has not been reported to naturally infect non-human animals and was hypothesized to have emerged from the mRNA of a HBV-infected human (6), although other origins involving viroids (circular, single-stranded RNA pathogens of plants) have also been speculated (7).

Recent discoveries have questioned the origins and mechanisms of deltavirus diversification. First, HDV-like genomes were detected in ducks, snakes, newts, toads, fish and termites (8-10). These agents were more closely related to one another than they were to their hosts' genomes, making independent emergence from each host mRNA unlikely. Furthermore, the absence of hepadnaviral co-infections suggested exploitation of diverse helpers, a finding supported by experimental evidence that HDV can be disseminated via a variety of non-HBV enveloped viruses (11). The presence of deltaviruses in non-human hosts could arise from historical co-speciation with hosts and/or helpers. This hypothesis predicts that deltaviruses should be more pervasive in nature than is currently appreciated. However, most will have likely remained undetected given that all discoveries of non-human deltaviruses to date used

bioinformatic interrogation of untargeted metagenomic sequence data, an approach that remains sparsely applied in most animal taxa (12,13). Alternatively, helper and host plasticity could enable deltavirus diversification by host switching. However, assuming non-human deltaviruses have similar biology to HDV, host switching would require overcoming an evolutionary challenge that would be unique among animal pathogens: either simultaneously shifting with their established helpers or co-opting pre-existing viruses in the recipient host species. Whether the host distribution of deltaviruses reflects a co-evolutionary process or occasional transmission among distantly related hosts remains untested.

Bats (Chiroptera) are a globally distributed mammalian order whose frequent association with zoonoses has led to the generation of an exceptional number of metagenomic datasets which might reveal uncharacterized deltaviruses. Bats also host diverse enveloped viruses, including hepadnaviruses (14), which might mobilize bat-associated deltaviruses. We hypothesized that if deltaviruses co-speciate with their hosts and/or helper viruses, bats should host diverse deltaviruses which transmit using hepadnaviruses, akin to the only currently known mammalian deltavirus (HDV). Alternatively, presence in a limited number of bat species, evidence of deltavirus transmission between bat species, incongruence between deltavirus and host phylogenies, or use of divergent helper viruses among mammal-infecting deltaviruses would each comprise independent lines of evidence for diversification by host and potentially helper virus shifting.

Results

We searched for deltaviruses in 259 previously published metagenomic datasets that were designed for viral discovery in bats, including 68 we generated (Table S1, S2). The full dataset

included sequences derived from feces, rectal swabs, urine, oropharyngeal/saliva swabs and organs. In total, samples represented at least 6,165 individual bats, spread across 30 genera from 11 countries. Reads homologous to deltaviruses were detected exclusively in saliva and rectal swabs from common vampire bats (*Desmodus rotundus*), a widely distributed Latin American sanguivore (Fig 1; Table S3). Four complete deltavirus genomes assembled from saliva metagenomes revealed two distinct genotypes (hereafter, DrDV-A and DrDV-B) that shared only 48.4-48.6% genome-wide nt identity (Table S4, S5). DrDVs resembled HDV in having circular genomes of 1,692 to 1,694 nt (circularity was confirmed by metagenomic reads spanning both ends of the linearized genome), containing an open reading frame encoding the small delta antigen protein (DAg; 194-196 amino acids [aa]), relatively high genome-wide GC levels (53.8-55%), high intramolecular base pairing (73.8-75.3%), and the presence of genomic and antigenomic ribozymes (Table S4, Fig S1, S2). In HDV, the large delta antigen protein (L-HDAg) is produced by RNA editing of the UAG stop codon to include 19 additional aa (15) and contains a farnesylation site which interacts with HBV (16). The DrDV-B DAg from the genome from bat colony CAJ1 terminated in UAG, which if edited similarly to HDV would generate a putative L-DAg containing an additional 28 aa (Fig S2). In contrast, DrDV-B DAg from the two other bat colonies from which genomes were sequenced (LMA6 and AYA11), as well as DrDV-A DAg, terminated in a UAA stop codon so would not appear to be similarly edited, although it is possible to extend the open reading frames through frameshifting (8). Importantly, no putative vampire bat L-DAg generated through either RNA editing or frameshifting contained a farnesylation site.

To understand the ecology and infection biology of DrDVs in wild bat populations, we analyzed saliva and blood samples collected in 2016-2017 from 12 vampire bat colonies across 3

regions of Peru. Using an RT-PCR targeting the DAg coding region, DrDV-A was detected in the saliva of a single adult female (bat 8299, N=240 screened) which was included in the metagenomic pool that first detected this genotype (Table S6, S7). Screening a smaller set of blood samples (N=60, including bat 8299) revealed no additional DrDV-A infections. In contrast, DrDV-B was detected in the saliva of 17.1% of bats (Fig 2A, N=240 screened, 0-35% infected across colonies). Prevalence varied neither by region of Peru (Likelihood ratio test; χ^2 = 3.21; d.f. = 2; P = 0.2) nor by bat age or sex (binomial generalized linear mixed model, Age: P = 0.38; Sex: P = 0.87), suggesting geographically widespread infection across demographic groups. Given that vampire bats subsist on blood, deltaviruses in bat saliva might represent contamination from infected prey. However, DrDV-B was detected in the blood of 6 out of 41 bats that were DrDV-B negative and in 4 out of 18 bats that had detectable DrDV-B in saliva. DrDV-B sequences were identical in the 4 individuals with paired positive saliva and blood samples, consistent with systemic infections (Fig 2B; Table S7). Significant spatial clustering of DrDV-B sequences at both the colony and regional levels indicated localized transmission cycles in bats (Fig 2C; Table S8).

To evaluate whether the bat deltaviruses used a hepadnavirus helper, we tested DrDV-positive and negative saliva (N=54) and blood samples (N=119) for hepadnaviruses using PCR (14) and comprehensively searched all metagenomic sequences from vampire bats for hepadnaviruses (Table S1, S6, S7). Consistent with a previous study in neotropical bats (14), we found no evidence of vampire bat-infecting hepadnaviruses in any dataset. We were unable to determine DrDV helper(s) since the metagenomic datasets which detected DrDV included sequences from multiple individuals pooled during library preparation. Nonetheless, analyses of viral communities identified genera that were over-represented in DrDV positive sites relative to

DrDV negatives sites, though we emphasize that differences were often marginal and should be investigated in metagenomic and tissue tropism studies which are explicitly designed to identify helper viruses (Fig S3).

Under the satellite-host species co-speciation hypothesis, many mammals spanning the divergence between vampire bats and humans should host deltaviruses. As such, there is no co-evolutionary explanation for the apparent absence of deltaviruses from non-vampire bats. However, it is conceivable that deltaviruses circulating in South American bats might have been missed due to the underrepresentation of non-vampire species in previously published datasets or differences in the quality of datasets across studies. We therefore performed additional metagenomic sequencing of saliva samples from frugivorous, insectivorous, nectarivorous, and sanguivorous bats in Peru (9 pools from 59 individuals across 23 species). Sequencing effort was comparable to a positive control pool which detected DrDV-B, but we found no additional deltaviruses in non-vampire bats (Table S9). Our earlier observations showed that deltavirus RNA was detectable by RT-PCR in individual vampire bat samples which had been included in metagenomic sequencing pools which were negative for deltavirus (Table S3). We therefore used RT-PCR to screen saliva swabs from 87 non-*D. rotundus* bats, including individuals from metagenomic sequencing and individuals that were withheld to balance pool sizes (Fig S4). Consistent with the expected greater sensitivity of RT-PCR, we detected deltavirus RNA in a single *Carollia perspicillata* (N=31 *C. perspicillata* individuals tested). The partial DAg sequence obtained was genetically identical to a *D. rotundus* strain collected from the same roost (CAJ4), a result that was unlikely to be attributable to erroneous bat species assignment or laboratory contamination (Fig 2C, SI Text). Given the expected rapid evolutionary rate of deltaviruses (ca. 10^{-3} substitutions/site/year), this genetic identity is most parsimoniously

explained as spillover transmission from a vampire bat followed by an absence of or short-lived transmission among frugivorous bats at the time of sampling (17). Importantly, this finding strongly suggests that deltaviruses can transmit between host species on ecological timescales, which is a defining prerequisite for evolutionary diversification through host shifting.

Bayesian and Maximum Likelihood phylogenetic analyses of complete DAg amplified by RT-PCR (Table S6) revealed that DrDVs did not form a reciprocally monophyletic clade (Fig 3, Fig S5). Instead, DrDV-A shared a most recent common ancestor with HDV (posterior probability, PP = 1). The paraphyletic evolutionary relationship between bat deltaviruses was supported by uncorrected aa similarities which showed that the identity of DrDV-A to DrDV-B (49.9-58.7%) was generally less than the identity of DrDV-A to HDV (58.2-66.7% depending on genotype). In contrast, DrDV-B shared a most recent common ancestor with the snake deltavirus (PP = 0.75) despite being more similar to DrDV-A based on aa identity (Fig 3, Fig S5, Table S5). We suspect that long branch attraction arising from high inter-genotype divergence led to unreliable grouping of DrDV-B. Despite the uncertain phylogenetic placement of DrDV-B, HDV was more closely related by aa similarity to both DrDVs than it was to any other animal deltavirus (Table S5). Importantly, neither the placement of DrDV-A nor DrDV-B were consistent with monophyly of bat deltaviruses. In light of the absence of deltaviruses from other bat species, these results suggest that vampire bat deltaviruses most parsimoniously arose following two introductions into vampire bats from a yet undiscovered deltavirus reservoir in the Americas.

Discussion

Cross-species transmission is the primary source of newly emerging human diseases (18). Unlike conventional pathogens (e.g., viruses, bacteria, protozoans), the obligatory dependence of satellites on helper viruses creates a barrier to cross-species transmission that should promote host specificity. This study reports two novel deltaviruses from vampire bats - the first HDV-like satellites found in non-human mammals. Three lines of evolutionary and ecological evidence suggest that DrDVs are most likely to have arisen via transmission from other host species and have the potential to be transmitted from vampire bats to other species. Evolutionarily, paraphyly of bat viruses and the apparent absence of non-vampire bat deltaviruses is incompatible with co-speciation of deltaviruses with their hosts, but consistent with multiple introductions to bats. Ecologically, a putative cross-species transmission event from a vampire bat to a sympatric fruit bat and potentially a similar spillover infection into vampire bats (see below) demonstrate that satellites can overcome the initial barrier to host shifting: natural infection of additional animal species. Below we outline the relative support for competing mechanistic explanations of deltavirus host shifts and discuss potential implications of host shifting based on the ability of satellites to alter the behavior of their helper viruses.

Deltavirus host shifts could conceivably arise through several mechanisms. Mobilization without viral helpers (e.g., via intra-cellular bacteria or unaided spread by a currently unknown mechanism) is hypothetically possible but has never been described. Given that the best studied deltavirus (HDV) is helper-dependent and the evolutionarily related deltaviruses share a similar genomic structure suggestive of a similar life history strategy, helper virus mediated host switching seems a reasonable expectation. Satellite host shifting could be facilitated by existing helpers within the donor host or by acquiring novel helpers within the recipient host. If deltaviruses colonized new hosts using their existing helpers, we would expect DrDVs, like their

only mammal-infecting relative HDV, to have been associated with hepadnaviruses. Instead, hepadnavirus infections were apparently absent from DrDV-infected vampire bats. Although we cannot rule out the possibility that clearance of acute hepadnavirus infections left DrDVs stranded within cells, considerable search efforts here and elsewhere have failed to demonstrate the existence of vampire bat-infecting hepadnaviruses (14). The lack of farnesylation sites in DrDVs raises further doubts that hepadnaviruses facilitate transmission. Given the growing body of evidence that deltaviruses can exploit diverse enveloped viruses (11,19), concurrent or sequential swapping of host species and helper viruses seems the most plausible explanation for deltavirus diversification. Our results therefore imply a swap in helper use along the evolutionary divergence separating human and vampire bat-infecting deltaviruses, though the current helper(s) that facilitate transmission among bats remain a mystery.

The presence of two divergent and paraphyletic deltaviruses in vampire bats implies independent evolutionary origins. One introduction (DrDV-B) has evidently sustained transmission long enough to diverge into geographically distinct sub-lineages, which were readily detectable in vampire bat saliva and blood. In contrast, the rarity and geographic restriction of DrDV-A may indicate it as a nascent host shift or spillover infection. Alternatively, the tissue tropism of DrDV-A may limit detection in saliva and blood. The evolutionary origins of both DrDVs are necessarily speculative given that deltaviruses infecting mammals outside of humans were until now unknown. Given that deltaviruses were exclusively detected in vampire bats, we suggest that introductions may have been mediated by blood feeding on currently unknown, but likely non-bat, deltavirus reservoir(s) in the American neotropics. Dietary studies provide some clues which could direct efforts to identify the progenitors of DrDVs. Across the range of DrDV-B, vampire bats feed on a wide range of hosts, including livestock (e.g. cows,

pigs, goats, chickens), large-bodied wild mammals (e.g. deer, tapir, sea lions), and humans (20-22). In contrast, DrDV-A was detected at a high-elevation site (2947m) with few wild mammals, suggesting a livestock origin may be more likely. A human origin for DrDVs (i.e., reverse zoonosis) is ecologically plausible since phylogenetic studies and clinical records indicate that humans may have introduced HDV into South America as recently as the 1930s (23). However, because DrDVs do not descend from known HDV genotypes, a reverse zoonosis would imply that highly divergent human deltaviruses exist undetected in South America. Notably, the alternative possibility of a non-human, non-bat origin for DrDVs also requires that additional undiscovered deltaviruses exist in the Americas. Additional metagenomic studies in a variety of human and non-human hosts may clarify the origins of both DrDV and HDV.

Frequent detection of DrDV-B in vampire bat saliva implies that a wide diversity of species on which vampire bats feed are likely to be exposed to bat deltaviruses. Zoonotic transmission is a particular concern in areas where humans are frequently fed upon (24,25). Replication of DrDV in human cells would be unsurprising given the ability of the more divergent snake deltavirus to do so (19). Moreover, HDV infects woodchucks (a rodent) experimentally, suggesting that deltaviruses generally may have a broad cellular tropism (5). Human-infecting viruses which might facilitate zoonotic transmission of DrDV are also prevalent in high-risk areas; for example, HBV infects up to 85% of individuals in some Amazonian communities (26). However, without the farnesylation site thought to be required for HDV envelopment by HBV, DrDVs would need to interact with HBV through a different mechanism, making HBV-mediated zoonotic transmission unlikely to be efficient. Other geographically-relevant, blood-borne human viruses such as hepatitis C virus and dengue virus mobilize HDV under laboratory conditions and deserve further attention as candidate DrDV

helpers in the context of a human host (11). Although the evidence above indicates limited ecological, virus replication, or helper virus barriers, fully understanding the likelihood of zoonotic transmission requires experimental confirmation that DrDV replicates in human cells and is mobilized by human helper viruses. Given widespread evidence of recombination among HDV genotypes and the high prevalence of HDV in some human populations, *in vitro* studies should also evaluate the potential of DrDV to recombine with HDV (26-28). Intriguingly, if zoonotic transmission occurs, it is unlikely to have been detected since serological- or PCR-based surveillance are unlikely to detect divergent deltaviruses. The metagenomic and RT-PCR approaches we describe here would enable more robust detection of deltavirus infections.

More generally, our results suggest that an obligate hyper-parasite is able to jump between animal host species through an unusual process that requires parasitizing evolutionarily independent viruses. The proposed host and helper switching would mean that deltaviruses join a broader set of viruses or virus-like elements that transmit between host species despite existing through outwardly improbable life history strategies. For example, viroids do not encode proteins or rely on helper viruses but can transmit between some plant species (29), exemplifying an even simpler strategy than that of satellites. Multipartite viruses which package genome segments across multiple capsids can also shift between plant species despite requiring that capsids simultaneously coinfect a cell or groups of cells in the novel host (30-32). While the dependence of multipartite viruses on co-infecting segments is analogous to the satellite requirement for helper virus co-infections, the evolutionary strategies implicated are distinct. Whereas the segments of multipartite virus segments share a common origin and are all required for full functionality, akin to mutualistic cooperation, the satellite-helper interaction is parasitic in nature and involves evolutionarily unrelated entities (33). Although host shifting occurs in plant satellite

viruses (34,35), the helper mediated host-shifting strategy of deltaviruses we propose would be unique among animal-infecting pathogens. This implies that the dynamics of and constraints on satellite virus host shifts will differ from those of conventional multi-host animal pathogens. Specifically, given the broad cellular tropism of deltaviruses, satellite-helper interactions would likely be more important determinants of cross-species transmission than satellite-host interactions (11,19). Consequently, anticipating host shifts would require understanding the determinants of satellite and helper virus compatibility along with prevalence and diversity of compatible helpers in candidate hosts, rather than classical barriers such as host cell receptor use or evasion of innate immunity. Since satellites in general and HDV in particular are expected to alter the pathogenesis and transmissibility of their helper viruses (1), our findings imply the potential for deltaviruses to act as host-switching virulence factors that could alter the progression of multiple viral infections in multiple host species.

Materials and Methods

Bioinformatic screening of published metagenomic datasets

We bioinformatically screened 259 published bat viral metagenomic datasets (36-50) for deltaviruses (Table S1, S2, S9).

Metagenomic datasets generated at the University Glasgow (N=78, including 22 from (46), 46 from (47), and 10 new datasets, described below) were analyzed using an in-house bioinformatic pipeline (46). These datasets included samples from vampire and non-vampire bats from Peru. Briefly, after quality trimming and filtering, reads were analyzed by blastx using DIAMOND (51) against a RefSeq database to remove bacterial and eukaryotic reads. Remaining reads were then *de novo* assembled using SPAdes (52) and resulting contigs were analyzed by

blastx using DIAMOND against a non-redundant (NR) protein database (53). KronaTools (54) and MEGAN (55) were used to visualize and report taxonomic assignments.

Metagenomic datasets downloaded from online repositories (N=191) were processed using a pipeline designed specifically to detect HDV-like sequences. Raw reads were initially screened by blastx against a deltavirus-only DIAMOND database containing all human HDV protein sequences from GenBank, our novel DrDV sequences, and HDV-like sequences from snakes, ducks, amphibians, fish and termites. As the small size of this database was prone to false positive detections based on e-values, all potential deltavirus reads were subsequently analyzed by blastx using DIAMOND against a NR protein database. This deltavirus detection pipeline was validated using metagenomic libraries from vampire bats which were known to contain or not to contain deltaviruses based on the more comprehensive virus discovery pipeline described above.

We performed comprehensive viral discovery on several additional datasets to evaluate the presence of HBV-like helper viruses in metagenomic data. Wu *et al.* (43) noted that their datasets contained reads matching to *Hepadnaviridae*. We re-analyzed these datasets using our virus discovery pipeline and similarly recovered *Hepadnaviridae* reads, confirming our ability to detect HBV-like viruses if present. In addition to checking our own datasets for HBV-like reads using the virus discovery pipeline (described above), we used the same pipeline to comprehensively screen other published vampire bat datasets (44,48,49) and found no *Hepadnaviridae* reads, confirming the absence of HBV-like viruses in all vampire bat datasets analyzed.

DrDV genome detection and characterization

Genome sequences of DrDVs were produced by mapping cleaned reads back to the SPAdes-generated contigs within Geneious v 7.1.7 (56). Regions of overlapping sequence at the ends of genomes due to linear *de novo* assemblies of circular genomes were resolved manually. For nucleotide analyses, genomes were aligned using MAFFT v 7.017 (57) and percent nucleotide identities to other deltavirus genomes were obtained from Geneious. The amino acid sequence of the small delta antigen protein was extracted from sequences using getorf (58). Other smaller identified open reading frames did not exhibit significant homology when evaluated by protein blast against Genbank. Percent identities at the amino acid level were determined using Geneious and protein domain homology was analyzed using Hhpred (59). Ribozymes were identified manually by examining the region upstream of the delta antigen open reading frame where ribozymes are located in other deltavirus genomes (8,9). RNA secondary structure and self-complementarity were determined using the webservers for mFold (60) and RNAStructure (61).

Capture and sampling of wild bats

Initial virus discovery efforts in Peruvian vampire bats used metagenomic datasets generated from bats captured at 23 colonies between 2013 and 2016 (46,47). For studies on DrDV prevalence in vampire bats, we studied 12 focal sites in three departments of Peru between 2016-2017 (Fig 2A). Other bat species roosting in the same colonies were captured and sampled opportunistically, adding to an existing bank of historical samples from Peruvian bats. Age and sex of bats were determined as described previously (46). Saliva samples were collected by allowing bats to chew on sterile cotton-tipped wooden swabs (Fisherbrand). Blood was collected from vampire bats only by lancing the propatagial vein and saturating a sterile cotton-tipped

wooden swab with blood. Swabs were stored in 1 mL RNALater (Ambion) overnight at 4°C before being transferred to dry ice and stored in -70°C freezers.

Bat sampling protocols were approved by the Research Ethics Committee of the University of Glasgow School of Medical, Veterinary and Life Sciences (Ref081/15), the University of Georgia Animal Care and Use Committee (A2014 04-016-Y3-A5), and the Peruvian Government (RD-009-2015-SERFOR-DGGSPFFS, RD-264-2015-SERFOR-DGGSPFFS, RD-142-2015-SERFOR-DGGSPFFS, RD-054-2016-SERFOR-DGGSPFFS).

RNA extraction and metagenomic sequencing

Total nucleic acid was extracted from individual swabs on a Kingfisher Flex 96 automated extraction machine (ThermoFisher Scientific) with the Biosprint One-for-all Vet Kit (Qiagen) using a modified version of the manufacturer's protocol as described previously (46). Ten pools of nucleic acids from vampire bats and other bat species were created for shotgun metagenomic sequencing (Table S9). Eight pools comprised samples from bats in the same genus (2-10 individuals per pool depending on availability of samples, 30 μL total nucleic acid per individual). The CAJ1 vampire bat pool from (47) which contained deltavirus reads was included as a sequencing control. The final pool ("Rare species") comprised 8 other bat species that had only one individual sampled each. Pools were treated with DNAse I (Ambion) and purified using RNAClean XP beads (Agencourt) following (46). Libraries were prepared using the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Clontech) and sequenced on an Illumina NextSeq500 at The University of Glasgow Polyomics Facility. Samples were bioinformatically processed for viral discovery as described above, with a slight modification to the read trimming step to account for shorter reads and a different library preparation kit.

RT-PCR and sequencing of blood and saliva samples

Primers were designed to screen bat saliva and blood samples for a conserved region of the DAg protein of DrDV-A (236bp) and DrDV-B (231bp), by hemi-nested and nested RT-PCR respectively (Table S6). Alternative primers were designed to amplify the complete DAg for DrDV-A (707bp) and DrDV-B (948bp) using a one-step RT-PCR (Table S6). cDNA was generated from total nucleic acid extracts using the Protoscript II First Strand cDNA synthesis kit with random hexamers; RNA and random hexamers were heated for 5 minutes at 65°C then placed on ice. Protoscript II reaction mix and Protoscript II enzyme mix were added to a final concentration of 1x, and the reaction was incubated at 25°C for 5 minutes, 42°C for 15 minutes, and 80°C for 5 minutes. PCR was performed using Q5 High-Fidelity DNA Polymerase (NEB). Each reaction contained 1x Q5 reaction buffer, 200 μM dNTPs, 0.5 μM each primer, 0.02 U/μL Q5 High Fidelity DNA polymerase and either 2.5 μL cDNA or 1 μL Round 1 PCR product. Reactions were incubated at 98°C for 30 seconds, followed by 40 cycles of 98°C for 10 seconds, 61-65°C for 30 seconds (or 58-60°C for 30 seconds for the complete DAg), 72°C for 40 seconds, and a final elongation step of 72°C for 2 minutes. PCR products of the correct size were confirmed by re-amplification from cDNA or total nucleic acid extracts and/or Sanger sequencing (Eurofins Genomics).

Bat species confirmation

We confirmed the morphological species assignment of the *C. perspicillata* individual in which DrDV-B was detected by sequencing cytochrome B. Cytochrome B was amplified from the same saliva sample in which DrDV-B was detected using primers Bat 05A and Bat 04A (62) and

GoTaq Green Master Mix (Promega) according to the manufacturer's instructions, and the resulting product was Sanger sequenced (Eurofins Genomics) then evaluated by nucleotide blast against Genbank.

Hepadnavirus screening in Peruvian bats

We tested samples for the presence of bat hepadnavirus as a candidate helper virus to DrDV. DNA from saliva and blood samples was screened for HBV-like viruses using pan-*Hepadnaviridae* primers (HBV-F248, HBV-R397, HBV-R450a, HBV-R450b; Table S6) and PCR protocols (14). We used a plasmid carrying a 1.3-mer genome of human HBV that is particle assembly defective but replication competent as a positive control.

Statistical and phylogenetic analyses

We modeled the effects of age and sex on DrDV-B presence in saliva using a binomial generalized linear mixed model (GLMM) in the package lme4 (63) in R version 3.5.1 ((64). Age (female/male) and sex (adult/subadult) were modeled as categorical variables, with site included as a random effect. We also evaluated differences in DrDV-B prevalence between regions of Peru using a binomial generalized linear model (GLM), and used the *Anova* function of the *car* package (65) to calculate the likelihood ratio χ_2 test statistic.

To examine relationships among DrDV-B sequences, Bayesian phylogenetic analysis was performed on a 214bp fragment of the DAg. Sequences from saliva and blood of 41 *D. rotundus* and saliva from one *C. perspicillata* were aligned using MAFFT within Geneious. Duplicate sequences originating from the blood and saliva of the same individuals were removed. Alignments were trimmed using trimal (66) on the Phylemon server (67) with automatic

parameters, and the best model of sequence evolution was determined using jModelTest2 (68). Phylogenetic analysis was performed using MrBayes 3.6.2 (69) with the GTR+I model. The analysis was run for 4,000,000 generations and sampled every 2,000 generations, with the first 1,000 trees removed as burn-in. The association between phylogenetic relationships and location at both the regional and colony level was tested using BaTS (70) with 1,000 posterior trees and 1,000 replicates to generate the null distribution.

Bayesian and Maximum Likelihood phylogenetic analyses were performed on complete DAg amino acid sequences to place the novel DrDVs relative to HDV and other deltaviruses. Two alignments were generated. First, we created an 'all deltavirus' dataset including DrDV-A and DrDV-B sequences, reference genomes representing the eight clades of human HDV (71) and all novel deltaviruses (8-10). Second, we created a 'focused deltavirus' dataset by removing the most divergent taxa, leaving only human, bat, avian and snake deltaviruses. For both datasets, sequences were aligned using MAFFT within Geneious and trimmed using trimal with gappyout settings. The best model of sequence evolution was determined using ProtTest3 (72). Bayesian phylogenetic analysis was performed in MrBayes, specifying outgroups as termite deltavirus (all deltavirus dataset) or avian deltavirus (focused deltavirus dataset), the latter based on its placement in the phylogenetic analysis of the all deltavirus alignment. The analysis was run allowing for model jumping among all possible fixed rate models. The all deltavirus analysis was run for 5,000,000 generations and sampled every 2,500 generations, with the first 500 trees discarded as burn-in. The focused deltavirus analysis was run for 4,000,000 generations and sampled every 2,000 generations, with the first 1,000 trees discarded as burn-in. Maximum Likelihood phylogenetic analysis was performed on the all deltavirus dataset using RaxML (73) with the JTT+G+F substitution model and 1,000 bootstrap replicates. Trees were visualized in

FigTree (74); the focused deltavirus dataset was rooted using the avian deltavirus and the outgroup dropped for visualization using the *ape* package in R (75). We tested nucleotide alignments of the DAg for recombination with the program GARD (76) on the Datamonkey webserver (77), but found no evidence of recombination.

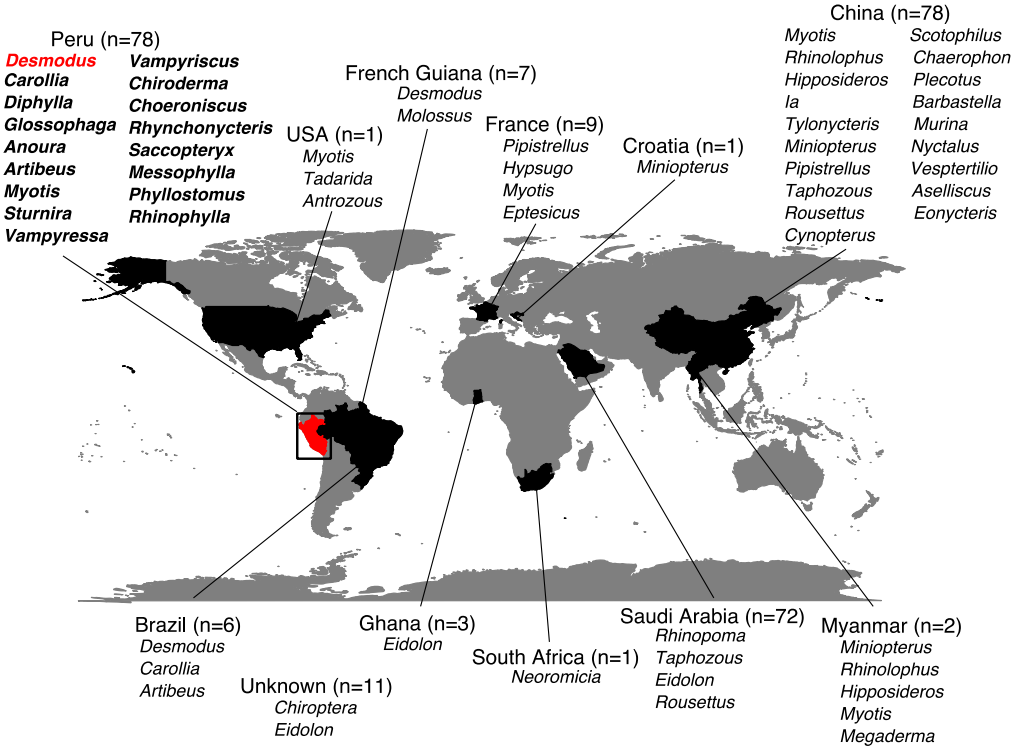

Fig 1. The known global diversity of bat deltaviruses is limited to common vampire bats. Previously published and newly generated metagenomic datasets were examined bioinformatically for the presence of deltavirus-like sequences. Countries screened are colored black, with the number of sequencing pools noted by each country name and the genera of included bats below. Bold indicates new dataset generated in this study; red indicates deltavirus detection.

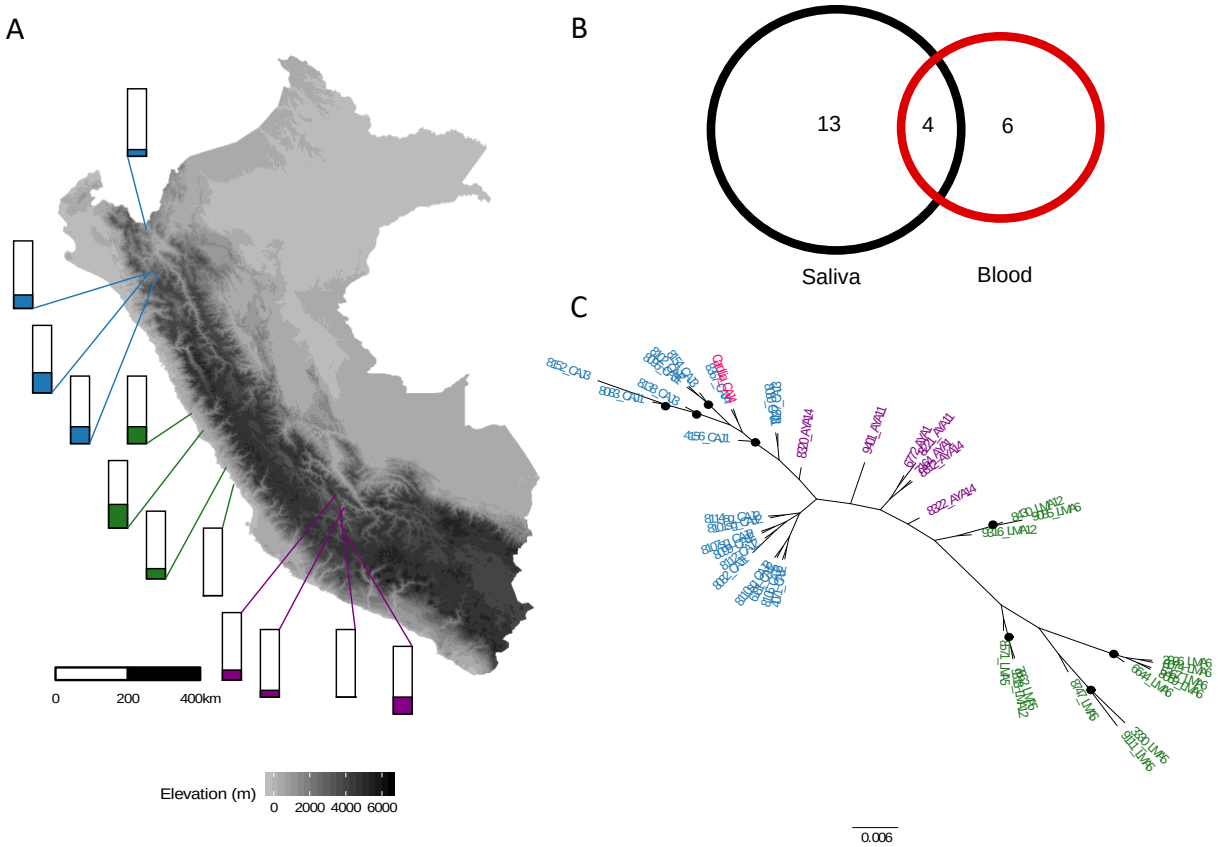

Fig 2. Prevalence and genetic structure of DrDV-B in Peruvian vampire bats. A) RT-PCR prevalence of DrDV-B in *Desmodus rotundus* colonies in Peru. Thermometers (N=20 for each) show the proportion of positive bats in each site; colors correspond to the three departments of Peru studied: Cajamarca (blue), Lima (green) and Ayacucho (purple). Elevation is shown in grayscale. B) Comparison of DrDV-B detections in saliva and blood. The four individuals in the center had genetically identical DrDV-B sequences in both saliva and blood. C) Bayesian phylogeny of a 214 nucleotide alignment the partial delta antigen region of DrDV-B sequenced from Peruvian bats. Taxon labels are colored by department as in panel A, and the deltavirus from the *Carollia* individual is shown in red. Nodes with posterior probability >0.9 are shown in black.

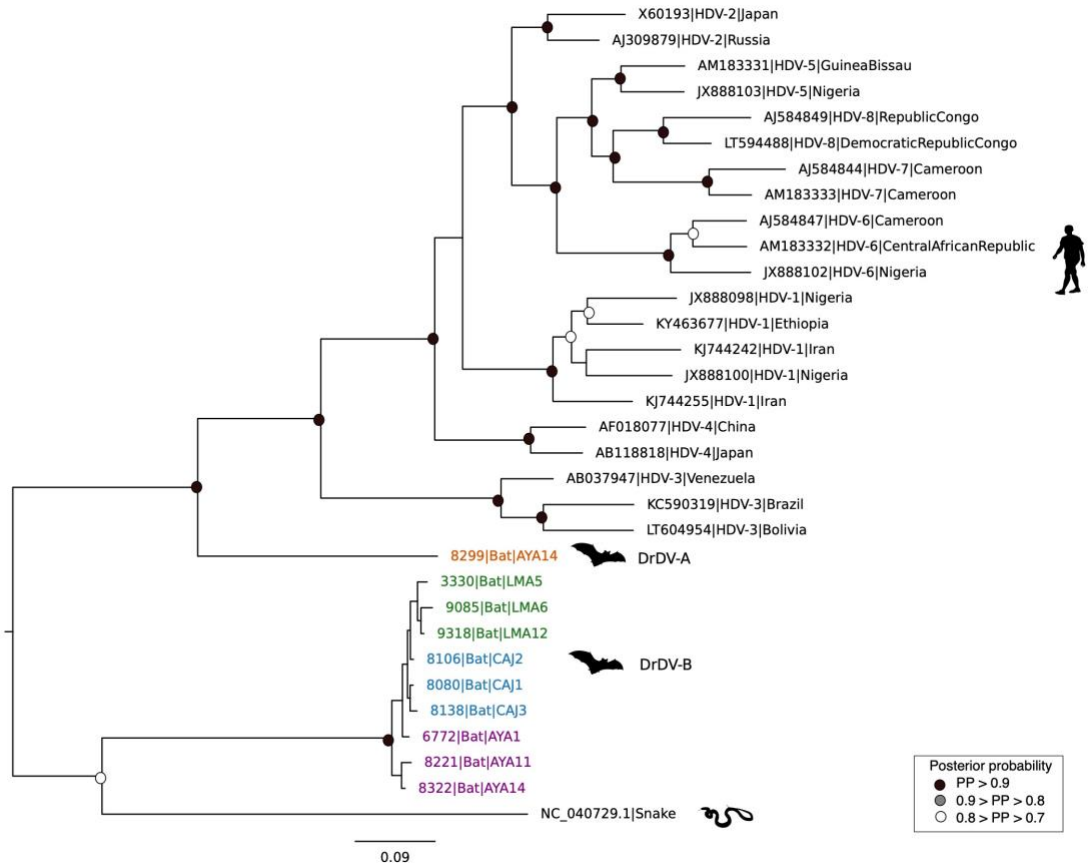

Fig 3. Evolutionary history of bat deltaviruses. Bayesian phylogeny of a 207 amino acid alignment of the complete delta antigen protein. DrDV-A (orange) and DrDV-B (green, blue and purple according to region) are shown along with representative sequences from each human HDV genotype and the snake deltavirus. The avian deltavirus was used to root the tree but is not depicted in the phylogeny.

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We thank Jaime Pacheco, Luigi Carrasco, Yosselym Luzon, Saori Grillo and Megan Griffiths for field and laboratory assistance, Joseph Hughes for analysis advice, and Ana da Silva Filipe, Felix Drexler and Pablo Murcia for comments on earlier versions of the results and manuscript.

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Supplementary Materials for

Satellite virus diversification through host shifting revealed by novel deltaviruses in vampire bats

Laura M. Bergner, Richard J. Orton, Alice Broos, Carlos Tello, Daniel J. Becker, Jorge E. Carrera, Arvind H. Patel, Roman Biek, Daniel G. Streicker

Supplementary Text

Putative cross-species transmission of DrDV-B to a frugivorous bat.

The detection of a vampire bat associated deltavirus in a frugivorous bat (*Carollia perspicillata)* is strongly suggestive of cross-species transmission but might also arise through mis-assignment of bat species in the field or contamination of samples during laboratory processing. To exclude the possibility of host species mis-identification, we confirmed morphological species assignment by sequencing Cytochrome B from the same saliva sample in which we amplified deltavirus (see Methods), which showed 99.49% identity with a published *C. perspicillata* sequence in Genbank (Accession AF511977.1). Laboratory contamination was minimized by processing all samples through a dedicated PCR pipeline with a one directional workflow. PCR reagents are stored and master mixes prepared in a laboratory that is DNA/RNA free, and which cannot be entered after going into any other lab. Field collected samples from bats are extracted and handled in a room strictly used for clinical samples which cannot be entered after going in any other lab aside from the master mix room. To further exclude laboratory contamination, we independently amplified the *C. perspicillata* deltavirus product from two separate batches of cDNA. We used only round 1 primers of a nested PCR to avoid detecting trace amounts of potential contamination; in vampire bats only 68% of individuals deemed positive after round 2 were also positive in round 1. Furthermore, in the laboratory, samples from other bat species were handled separately from samples collected from vampire bats, with extractions and PCRs being performed on different days. As discussed in the main text, the absence of genetic divergence from sympatric strains in *D. rotundus* indicates limited or no onward transmission of DrDV-B in *C. perspicillata*. Whether the *C. perspicillata* sustained an actively replicating infection is uncertain, although detection in a single round of PCR (which was true for only 68% of DrDV-positive vampire bats) implies an intensity of infection which could suggest DrDV replication in the recipient host, though this would require further testing to confirm. Definitively resolving the extent of DrDV-B replication could be achieved using a quantitative RT-PCR targeting the DrDV antigenome. Such assays do not currently exist and after the confirmatory testing above, in addition to metagenomic sequencing, we unfortunately would no longer have sufficient RNA available from the *C. perspicillata* bat to run such a test if it were available. In summary, we are confident that the individual in which the deltavirus was detected is a *C. perspicillata* and although we cannot definitively exclude laboratory contamination from a vampire bat sample, we believe the most likely explanation to be cross-species transmission in nature, though whether this represents an active infection remains uncertain.

Supplementary Figures

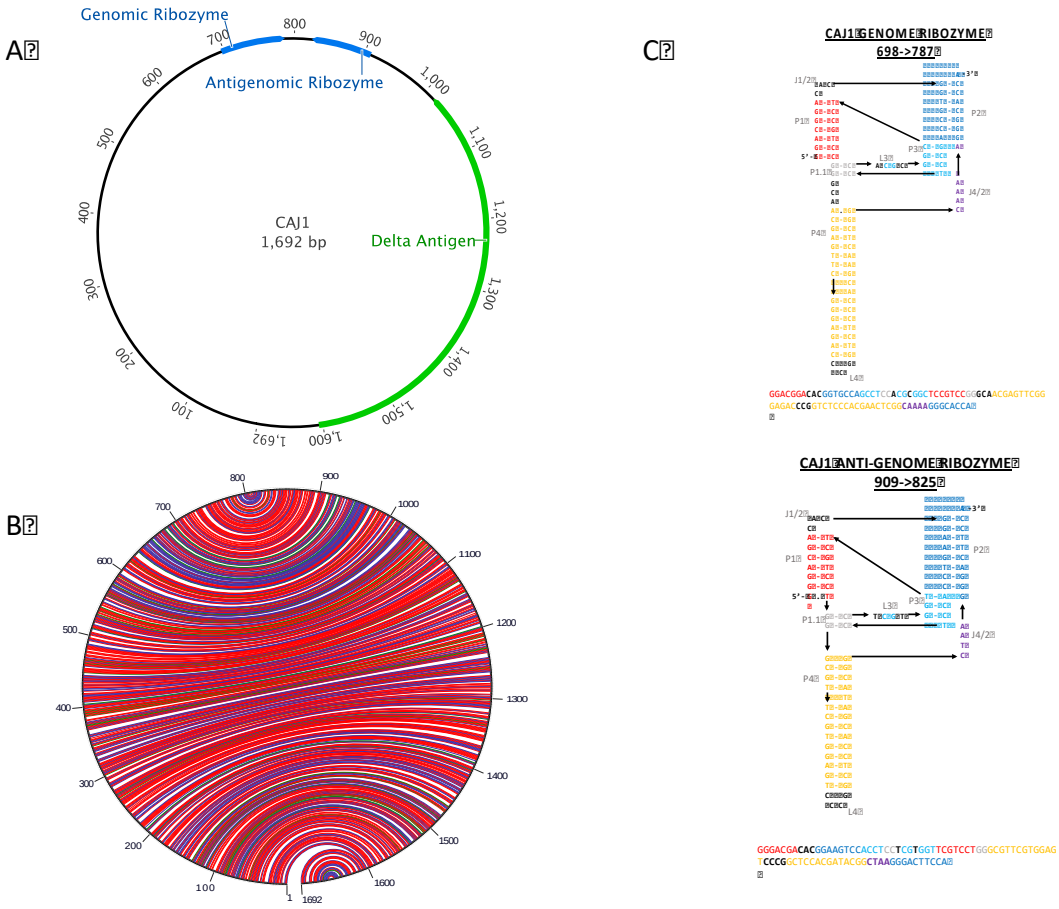


Fig. S1. DrDV genomes exhibit characteristics common to deltaviruses. (A) The locations of the delta antigen open reading frame (green) and genomic/antigenomic ribozymes (blue) are shown along the circular genome of an exemplar DrDV-B virus from site CAJ1. (B) Intramolecular base pairing of the same exemplar genome is depicted as lines connecting points on the circular genome – G-C pairs are red, A-U pairs are blue, G-U pairs are green, other pairs are yellow. (C) Genomic and antigenomic ribozyme secondary structures are shown along with genome location. Complementary regions are shown in the same color, and structures are depicted in the style of Webb & Luptak to facilitate comparison with ribozymes from previous studies (1-3).

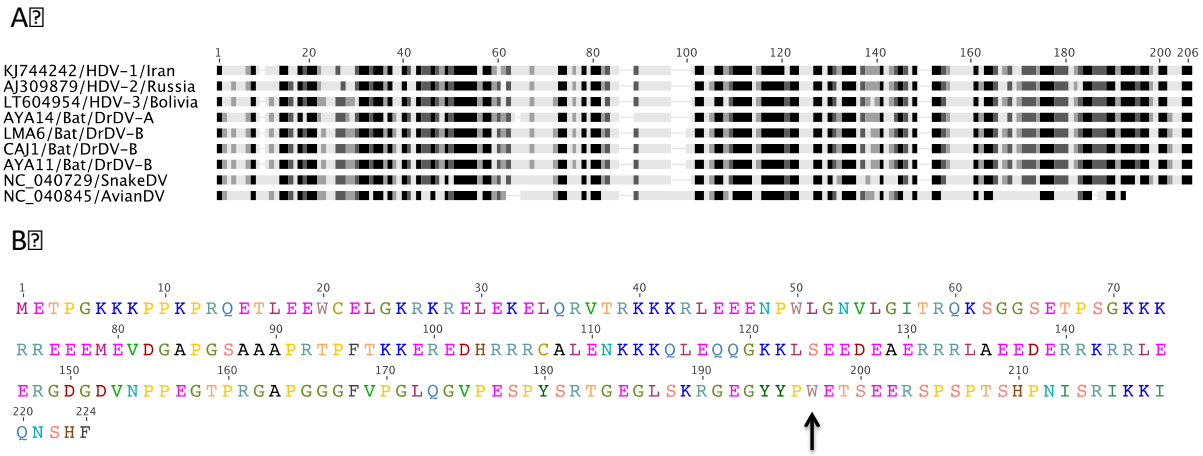


Fig. S2. Characterization of the DrDV delta antigen protein. (A) Alignment of delta antigen protein sequences for three HDV genotypes, new DrDV genomes, and snake and bird deltaviruses. Shading indicates level of similarity across all sequences, with regions of high identity in black and divergent regions in white. (B) Putative sequence of the DrDV large DAg for DrDV-B virus from the site CAJ1. The RNA editing site is marked with a black arrow; UAG has been edited to UGG yielding a tryptophan residue (W).

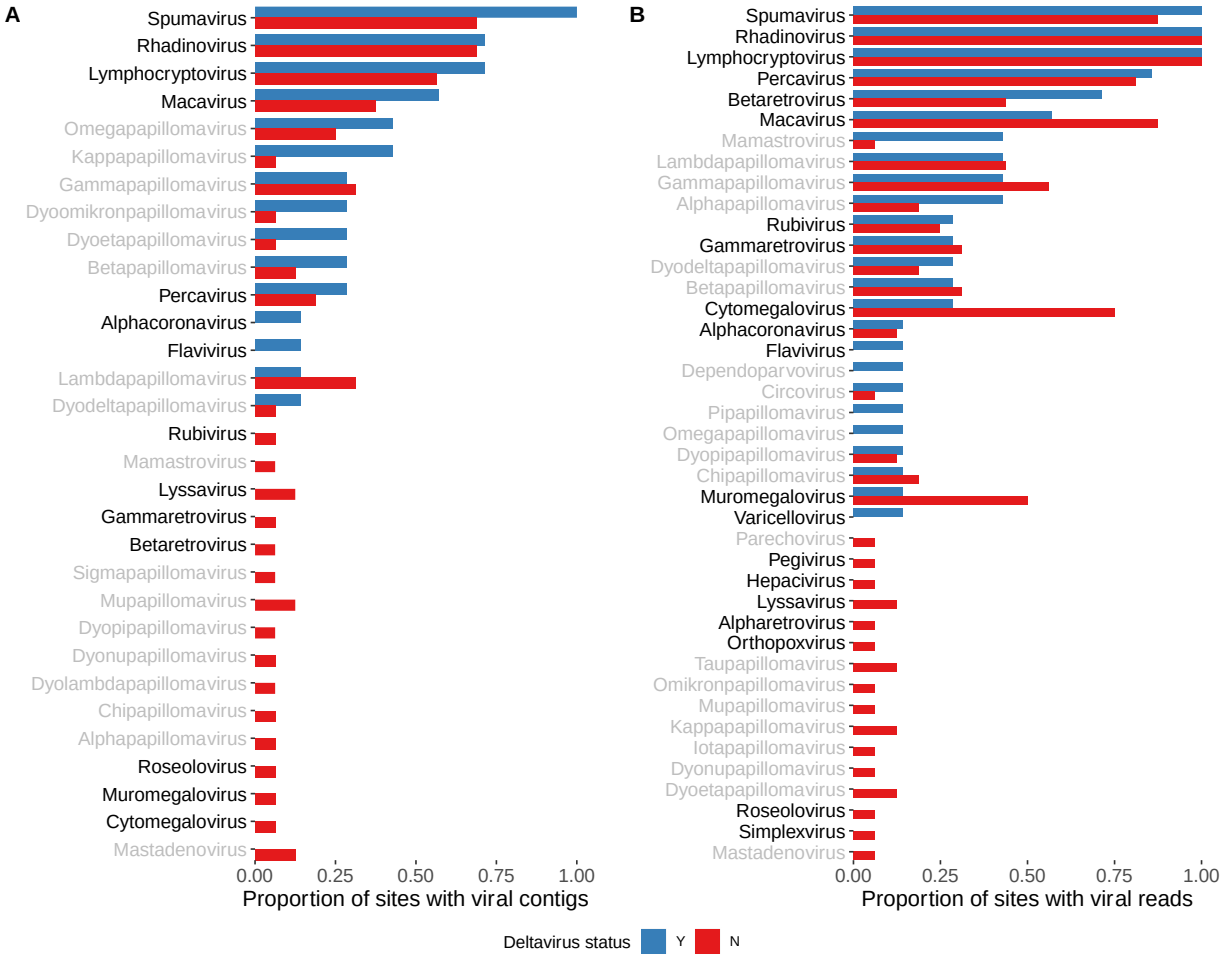


Fig. S3. Candidate helper viruses for DrDV as inferred from saliva metagenomic viral communities. Proportion of sites containing each viral genus as contigs (A) and reads (B) are shown for sites that were positive (blue) and negative (red) for DrDV. The pipeline used to generate candidate helper viruses involved first mapping raw reads against the vampire bat host genome, and then comparison to the NCBI RefSeq database to remove all bacterial and eukaryotic reads. Remaining reads were classified through comparison to the NCBI Viral RefSeq database. Enveloped viruses, which are more likely helper candidates, are shown in black while non-enveloped viruses are shown in gray.

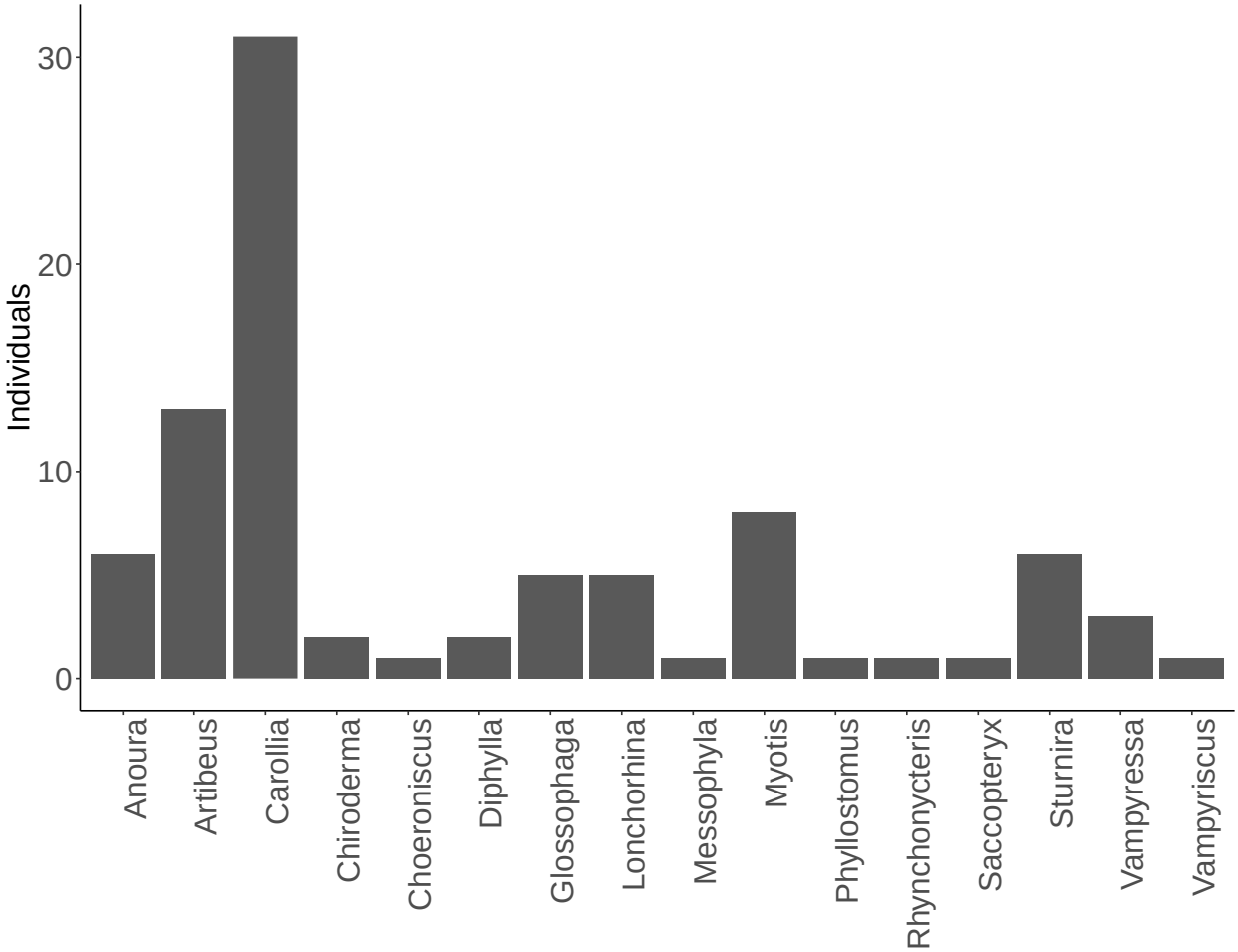
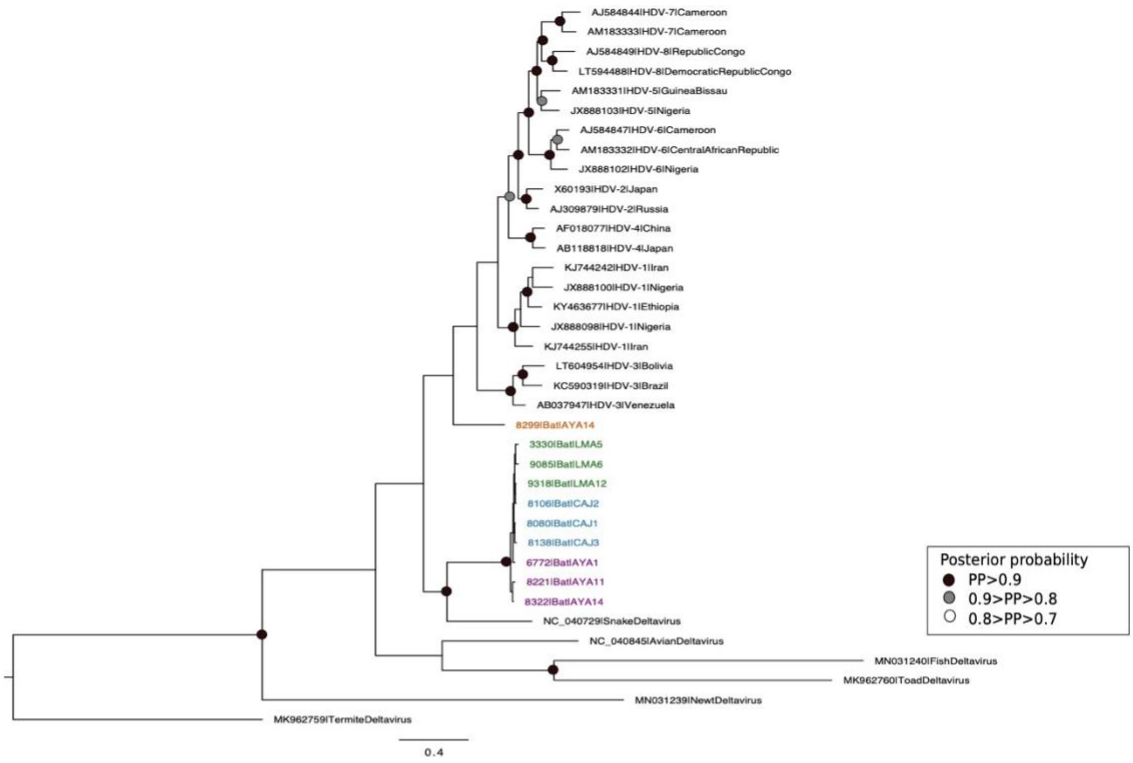
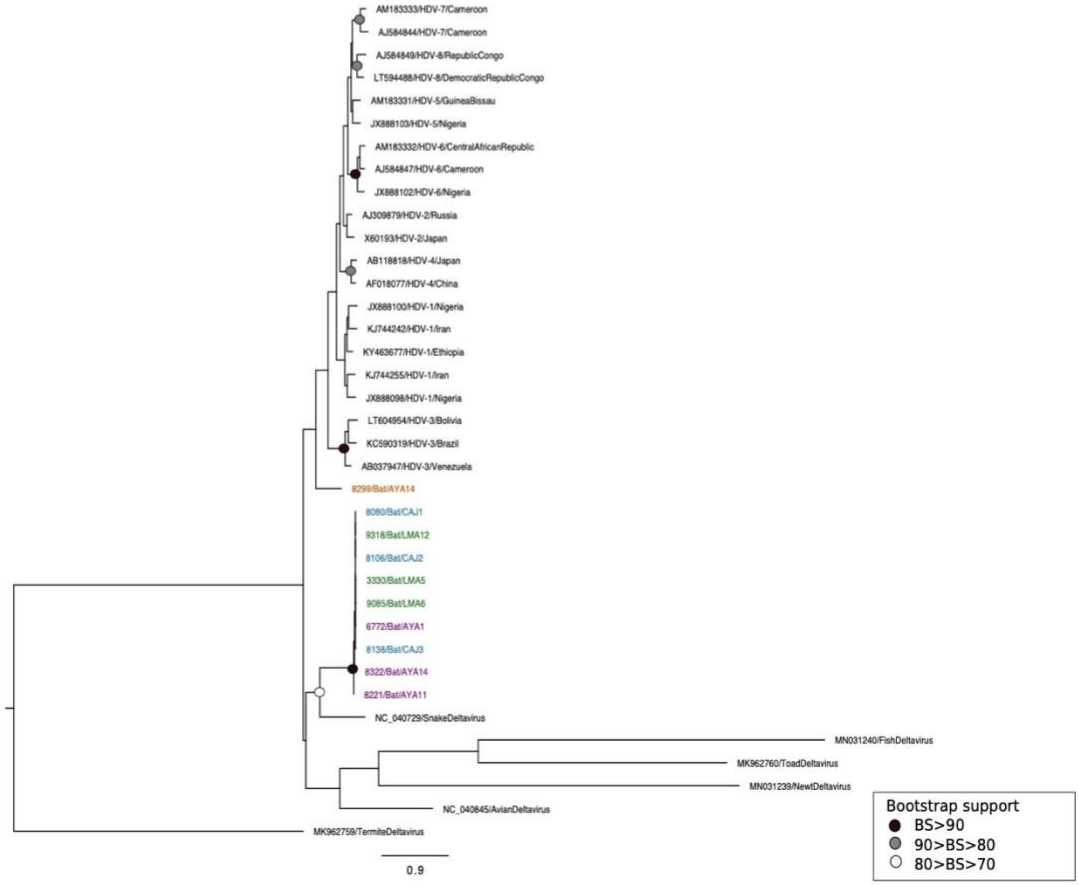


Fig. S4. Counts of non-*D. rotundus* bat species saliva swabs individually screened by RT-PCR for DrDV-B. Bars group bats by genus.

A


AJ584844|HDV-7|Cameroon
AM183333|HDV-7|Cameroon
AJ584849|HDV-8|RepublicCongo
LT594488|HDV-8|DemocraticRepublicCongo
AM183331|HDV-5|GuineaBissau
JX888103|HDV-5|Nigeria
AJ584847|HDV-6|Cameroon
AM183332|HDV-6|CentralAfricanRepublic
JX888102|HDV-6|Nigeria
X60193|HDV-2|Japan
AJ309879|HDV-2|Russia
AF018077|HDV-4|China
AB118818|HDV-4|Japan
KJ744242|HDV-1|Iran
JX888100|HDV-1|Nigeria
KY463677|HDV-1|Ethiopia
JX888098|HDV-1|Nigeria
KJ744255|HDV-1|Iran
LT604954|HDV-3|Bolivia
KC590319|HDV-3|Brazil
AB037947|HDV-3|Venezuela
8299|Bat|AYA14
3330|Bat|LMA5
9085|Bat|LMA6
9318|Bat|LMA12
8106|Bat|CAJ2
8080|Bat|CAJ1
8138|Bat|CAJ3
6772|Bat|AYA1
8221|Bat|AYA11
8322|Bat|AYA14
NC_040729|SnakeDeltavirus
NC_040845|AvianDeltavirus
MN031240|FishDeltavirus
MK962760|ToadDeltavirus
MN031239|NewtDeltavirus
MK962759|TermiteDeltavirus
0.4
Posterior probability
PP>0.9
0.9>PP>0.8
0.8>PP>0.7

B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JX888103/HDV-5/Nigeria
AM183332/HDV-6/CentralAfricanRepublic
AJ584847/HDV-6/Cameroon
JX888102/HDV-6/Nigeria
AJ309879/HDV-2/Russia
X60193/HDV-2/Japan
AB118818/HDV-4/Japan
AF018077/HDV-4/China
JX888100/HDV-1/Nigeria
KJ744242/HDV-1/Iran
KY463677/HDV-1/Ethiopia
KJ744255/HDV-1/Iran
JX888098/HDV-1/Nigeria
LT604954/HDV-3/Bolivia
KC590319/HDV-3/Brazil
AB037947/HDV-3/Venezuela
8299/Bat/AYA14
8080/Bat/CAJ1
9318/Bat/LMA12
8106/Bat/CAJ2
3330/Bat/LMA5
9085/Bat/LMA6
6772/Bat/AYA1
8138/Bat/CAJ3
8322/Bat/AYA14
8221/Bat/AYA11
NC_040729/SnakeDeltavirus
MN031240/FishDeltavirus
MK962760/ToadDeltavirus
MN031239/NewtDeltavirus
NC_040845/AvianDeltavirus
MK962759/TermiteDeltavirus
0.9
Bootstrap support
BS>90
90>BS>80
80>BS>70

Fig. S5. Phylogenetic analysis of DrDVs in the broader context of representative viruses from each HDV genotype and divergent deltaviruses. (A) Bayesian and (B) Maximum Likelihood analyses were performed based on a 193 amino acid alignment of the delta antigen protein for the all deltavirus dataset.

Supplementary Tables

Table S1.

Bat viral metagenomic datasets which were bioinformatically screened for deltavirus-like sequences.

ENA Project ID	Pools	Screen	Citation	Sample type(s)
SRP002405	1	DV database	(4)	Feces
PRJDB31	1	DV database	(5)	Feces
PRJEB659	3	DV database	(6)	Urine; Throat swabs; Lung tissue
PRJNA171017	2	DV database	(7)	Organs
PRJNA230690	9	DV database	(8)	Organs
PRJNA218570	1	DV database	(9)	Rectal swabs
PRJNA224954	1	DV database	(10)	Oral and rectal swabs
SRP011912	75	Community	(11)	Oral and rectal swabs
PRJNA341472	7	Community	(12)	Feces and oral swabs
PRJNA396802	1	DV database	(13)	Feces and rectal swabs
PRJEB28138	22	Community	(14)	Oral and rectal swabs
PRJEB34487	46	Community	(15)	Oral and rectal swabs
PRJNA480298	6	Community	(16,17)	Organs
PRJNA487099	72	DV database	(18)	Feces and rectal swabs
PRJEB2810	11	DV database	-	-
PRJNA433098	1	DV database	-	Feces
PRJEB35111	10	Community	This study	Oral swabs

Table S2.

Bat viral metagenomic datasets showing number of individuals for each species within each study.

ENA Project ID	Location	Genus	Species	Individuals
SRP002405	USA	*Myotis*	*sp*	-
SRP002405	USA	*Tadarida*	*brasiliensis*	-
SRP002405	USA	*Antrozous*	*pallidus*	-
PRJDB31	China	*Myotis*	*ricketti*	-
PRJDB31	China	*Scotophilus*	*kuhlii*	-
PRJDB31	China	*Hipposideros*	*armiger*	-
PRJDB31	China	*Myotis*	*sp*	-
PRJEB659	Ghana	*Eidolon*	*helvum*	-
PRJNA171017	Myanmar	*Miniopterus*	*fuliginosus*	640
PRJNA171017	Myanmar	*Rhinolophus*	*ferrumequinum*	176
PRJNA171017	Myanmar	*Hipposideros*	*armiger*	8
PRJNA171017	Myanmar	*Myotis*	*chinensis*	11
PRJNA171017	Myanmar	*Megaderma*	*lyra*	6
PRJNA171017	Myanmar	*Hipposideros*	*fulvus*	12
PRJNA230690	France	*Pipistrellus*	*pipstrellus*	5
PRJNA230690	France	*Hypsugo*	*savii*	1
PRJNA230690	France	*Myotis*	*nattereri*	1
PRJNA230690	France	*Myotis*	*mystacinus*	1
PRJNA230690	France	*Eptesicus*	*serotinus*	1
PRJNA218570	China	*Rhinolophus*	*ferrumequinum*	77
PRJNA218570	China	*Rhinolophus*	*affinus*	11
PRJNA218570	China	*Rhinolophus*	*hipposideros*	11
PRJNA218570	China	*Myotis*	*daubentonii*	86
PRJNA218570	China	*Myotis*	*davidi*	83
PRJNA224954	China	*Taphozous*	*melanopogon*	3
PRJNA224954	China	*Hipposideros*	*cineraceus*	1
PRJNA224954	China	*Hipposideros*	*armiger*	1
PRJNA224954	China	*Eonycteris*	*spelaea*	1
PRJNA224954	China	*Rousettus*	*leschenaulti*	1
SRP011912	China	*Myotis*	*ricketti*	258
SRP011912	China	*Myotis*	*myotis*	76
SRP011912	China	*Myotis*	*altarium*	75
SRP011912	China	*Myotis*	*daubentonii*	119
SRP011912	China	*Myotis*	*ikonnikovi*	68
SRP011912	China	*Myotis*	*davidi*	12
SRP011912	China	*Myotis*	*formosus*	8
SRP011912	China	*Myotis*	*sp*	136
SRP011912	China	*Myotis*	*siligorensis*	40

SRP011912	China	*Myotis*	*pequinius*	42
SRP011912	China	*Myotis*	*brandti*	24
SRP011912	China	*Rhinolophus*	*ferrumequinum*	659
SRP011912	China	*Rhinolophus*	*sinicus*	366
SRP011912	China	*Rhinolophus*	*affinus*	146
SRP011912	China	*Rhinolophus*	*pearsoni*	84
SRP011912	China	*Rhinolophus*	*pusillus*	290
SRP011912	China	*Rhinolophus*	*lepidus*	42
SRP011912	China	*Rhinolophus*	*macrotis*	86
SRP011912	China	*Rhinolophus*	*rex*	16
SRP011912	China	*Rhinolophus*	*hipposideros*	48
SRP011912	China	*Rhinolophus*	*rouxi*	42
SRP011912	China	*Hipposideros*	*armiger*	168
SRP011912	China	*Hipposideros*	*larvatus*	28
SRP011912	China	*Hipposideros*	*pomona*	80
SRP011912	China	*Hipposideros*	*cineraceus*	40
SRP011912	China	*Hipposideros*	*pratti*	130
SRP011912	China	*Ia*	*io*	128
SRP011912	China	*Tylonycteris*	*robustula*	20
SRP011912	China	*Tylonycteris*	*pachypus*	60
SRP011912	China	*Miniopterus*	*schreibersii*	240
SRP011912	China	*Pipistrellus*	*sp*	114
SRP011912	China	*Taphozous*	*melanopogon*	66
SRP011912	China	*Rousettus*	*leschenaulti*	128
SRP011912	China	*Cynopterus*	*sphinx*	179
SRP011912	China	*Chaerophon*	*plicata*	26
SRP011912	China	*Plecotus*	*auritus*	46
SRP011912	China	*Barbastella*	*beijingensis*	98
SRP011912	China	*Murina*	*leucogaster*	106
SRP011912	China	*Nyctalus*	*velutinus*	30
SRP011912	China	*Vespertilio*	*superans*	85
SRP011912	China	*Aselliscus*	*stoliczkanus*	31
PRJNA341472	French Guiana	*Desmodus*	*rotundus*	-
PRJNA341472	French Guiana	*Molossus*	*molossus*	-
PRJNA396802	South Africa	*Neoromicia*	*capensis*	36
PRJNA396802	South Africa	*Neoromicia*	*zuluensis*	4
PRJNA396802	South Africa	*Neoromicia*	*nana*	12
PRJNA396802	South Africa	*Neoromicia*	*cf.helios*	6
PRJEB28138	Peru	*Desmodus*	*rotundus*	109
PRJEB34487	Peru	*Desmodus*	*rotundus*	274
PRJNA480298	Brazil	*Desmodus*	*rotundus*	3
PRJNA480298	Brazil	*Carollia*	*perspicillata*	2
PRJNA480298	Brazil	*Artibeus*	*lituratus*	1

PRJNA487099	Saudi Arabia	*Rhinopoma*	*hardwickii*	29
PRJNA487099	Saudi Arabia	*Taphozous*	*perforatus*	17
PRJNA487099	Saudi Arabia	*Eidolon*	*helvum*	24
PRJNA487099	Saudi Arabia	*Rousettus*	*aegyptiacus*	2
PRJEB2810	Unknown*	*Chiroptera*†	-	-
PRJEB2810	Unknown*	*Eidolon*	*helvum*	-
PRJNA433098	Croatia	*Miniopterus*	*schreibersii*	-
PRJEB35111	Peru	*Carollia*	*perspicillata*	10
PRJEB35111	Peru	*Glossophaga*	*soricina*	5
PRJEB35111	Peru	*Desmodus*	*rotundus*	10
PRJEB35111	Peru	*Diphylla*	*ecaudata*	2
PRJEB35111	Peru	*Anoura*	*geoffroyi*	5
PRJEB35111	Peru	*Anoura*	*peruana*	1
PRJEB35111	Peru	*Artibeus*	*lituratus*	2
PRJEB35111	Peru	*Artibeus*	*obscurus*	3
PRJEB35111	Peru	*Artibeus*	*planirostris*	2
PRJEB35111	Peru	*Artibeus*	*fraterculus*	3
PRJEB35111	Peru	*Myotis*	*oxyotus*	6
PRJEB35111	Peru	*Myotis*	*sp*	2
PRJEB35111	Peru	*Sturnira*	*erythromos*	5
PRJEB35111	Peru	*Sturnira*	*sp*	1
PRJEB35111	Peru	*Vampyressa*	*sp*	2
PRJEB35111	Peru	*Vampyriscus*	*bidens*	2
PRJEB35111	Peru	*Chiroderma*	*trinitatum*	1
PRJEB35111	Peru	*Chiroderma*	*salvini*	1
PRJEB35111	Peru	*Choeroniscus*	*minor*	1
PRJEB35111	Peru	*Rhynchonycteris*	*naso*	1
PRJEB35111	Peru	*Saccopteryx*	*bilineata*	1
PRJEB35111	Peru	*Messophyla*	*macconelli*	1
PRJEB35111	Peru	*Phyllostomus*	*discolor*	1
PRJEB35111	Peru	*Rhinophylla*	*pumilio*	1

* Locations not provided for these datasets
† Bat species not specified
- Number of individuals in pool not specified

Table S3.

Contigs and reads homologous to deltavirus in viral metagenomic pools of feces (F) and saliva (SV) from vampire bats in Peru.

Region	DV contigs	DV reads	Colony	DV contigs	DV reads
AAC_H_F	0	0	AMA7_F	0	0
AAC_H_SV*	1	16	AMA7_SV	0	0
AAC_L_F	0	0	AMA2_F	0	0
AAC_L_SV	0	0	AMA2_SV	0	0
AMA_L_ F_NR	0	0	API1_F	0	0
AMA_L_F_R	0	0	API1_SV	0	0
AMA_L_SV	0	0	API17_F	0	0
CAJ_L_F_NR	0	0	API17_SV	0	0
CAJ_L_F_R	0	0	API140_F	1	6
CAJ_L_SV	0	0	API140_SV	0	0
CAJ_H_F_1	0	0	API141_F	0	0
CAJ_H_F_2	0	0	API141_SV	0	0
CAJ_H_SV*	1	17	AYA1_F	0	0
HUA_H_F	0	0	AYA1_SV	0	0
HUA_H_SV	0	0	AYA7_F	0	0
LMA_L_F_NR	0	0	AYA7_SV	1	3
LMA_L_F_R	0	0	AYA11_F	0	0
LMA_L_SV_NR	1	7	AYA11_SV*	1	52
LMA_L_SV_R*	1	47	AYA12_F	0	0
LR_L_F_NR	0	0	AYA12_SV	0	1
LR_L_F_R	0	0	AYA14_F	0	0
LR_L_SV	0	0	AYA14_SV*	1	35
			AYA15_F	0	0
			AYA15_SV	0	0
			CAJ1_F	0	0
			CAJ1_SV*	1	20
			CAJ2_F	0	2
			CAJ2_SV†	0	0
			CAJ4_F	0	0
			CAJ4_SV†	0	0
			CUS8_F	0	0
			CUS8_SV	0	0
			HUA1_F	0	0
			HUA1_SV	0	0
			HUA2_F	0	0
			HUA2_SV	0	0
			HUA3_F	0	0
			HUA3_SV	0	0

	HUA4_F	0	0
	HUA4_SV	0	0
	LMA5_F	0	0
	LMA5_SV	0	1
	LMA6_F	0	0
	LMA6_SV*	1	23
	LR2_F	0	0
	LR2_SV	0	0
	LR3_F	0	0
	LR3_SV	0	0

* Pools in which full deltavirus genomes were detected
† Pools in which DrDV was detected in the saliva of one or more individuals in the pool by RT-PCR, but were negative for deltavirus detection through metagenomics

Table S4.

Summary statistics for bat deltavirus genomes and protein domain homology analysis of predicted DrDV small delta antigens from saliva metagenomic pools.

Site	Lineage	Genome (nt)	GC content (%)	Intramolecular base pairing (%)	Delta antigen (aa)	Hhpred top hit	Probability top hit	e-value	Identity top hit (%)
AYA14	DrDV-A	1694	55	73.8	194	Oligomerization domain of hepatitis delta antigen	99.86	2.8e-25	59
AYA11	DrDV-B	1692	54.3	75.3	196		99.86	5.60E-25	45
CAJ1	DrDV-B	1692	53.8	74.3	196		99.86	5.40E-25	45
LMA6	DrDV-B	1694	54.3	74.6	196		99.85	8.30E-25	45

Table S5.

Genetic distances matrices showing representative deltavirus sequences with percent nucleotide identities between genomes (upper triangle) and percent amino acid identities between complete DAg sequences (lower triangle).

	HDV1	HDV2	HDV4	HDV5	HDV6	HDV7	HDV8	HDV3	DrDVA	DrDVB	SnakeDV	AvianDV	ToadDV*	FishDV*	NewtDV*
KJ744242/HDV1		72.5	68.4	65.8	67.1	67.3	66.1	60.4	53.6	46.4	42.4	37.8			
AJ309879/HDV2	72.4		75.8	74.4	73.4	71.9	73	61.2	53.6	46.5	43.5	37.4			
AB118818/HDV4	70.9	78.5		71.4	70.8	71.4	71.1	61.6	55.9	47.2	43.3	37.7			
JX888103/HDV5	70.5	79.9	74.8		70.6	70.2	71.6	59.3	52	46.9	42.9	37.9			
JX888102/HDV6	71.4	72	66.8	72.5		71	71.4	62.3	54.4	47.7	43.2	37.1			
AM183333/HDV7	69	72.6	72.1	75.4	75.4		74.5	59.6	52.4	46.3	43.7	38.3			
AJ584849/HDV8	68.4	75.4	72.8	79	71.8	74		58.9	52.9	45.8	41.7	37.1			
LT604954/HDV3	66.3	60.5	58.4	57.3	60.9	60.1	62.2		52.4	46.4	42.7	36.2			
AYA14/DrDVA	59.7	61.0	62.9	60.0	60.0	59.6	59.0	58.5		48.6	43.7	38.1			
LMA6/DrDVB	50.8	54.1	52.3	51.6	54.1	50.1	52.6	53.6	57.0		44.6	37			
NC_040729/SnakeDV	49.5	46.2	46.7	45.3	48.7	46.9	47.2	49	52.3	53.5		37.7			
NC_040845/AvianDV	36.7	36.9	36.4	36.4	36.4	37.5	36.4	37.2	37.4	38.6	35.6				
MK962760/ToadDV*	19.1	20.2	20.7	18.7	19.2	20.8	18.7	18.6	16.6	15.5	17.3	17.5			
MN031240/FishDV*	20.2	19.8	20.3	20.9	20.3	18.3	19.3	19.7	20.8	23.4	19.5	18.2	16.7		
MN031239/NewtDV*	21.2	22.2	20.9	20	20	18.8	24	17.7	19.9	18.1	18.8	18.7	15.7	16.2	
MK962759/TermiteDV*	18.1	17.6	19.2	18.2	20.7	19.8	18.2	20.1	19.2	19.2	18.2	15	11.9	17.5	14.3

*Divergent deltavirus genomes including newt, toad, fish and termite were not aligned at the nucleotide level

Table S6.

Primers used to screen samples for DrDV by RT-PCR and HBV by PCR.

Primer	PCR Round	Sequence (5´-3´)
DrDV-A		
DrDV_F1_GenoA	1&2	AGGGGTCTTTTTGGGAAATT
DrDV_R1_GenoA	1	AAGAAGAAGCAACTATCCGG
DrDV_R2_GenoA	2	CATCCAAGAGACCAAGAGAG
DrDV-B		
DrDV_F1_GenoB	1	TTCCCTTGYTGCTCCAGTTG
DrDV_R1_GenoB	1	CGGTAAGAAGAAACCTCCAA
DrDV_F2_GenoB	2	CCAGTTGTTTCTTCTTGTTCTC
DrDV_R2_GenoB	2	AAAAAGAAAGAGAGAACTGGAAAAA
DrDV Delta Antigen		
DeltaAntigenF1_GenoB	1	TCTGGTCTTATCTTTCTTACCTTAT
DeltaAntigenR1_GenoB	1	AAACCTTCCTTTATTCTATTTCGAA
DeltaAntigenR1_GenoA	1	CCTTTACCTTTAATTCTCTTGGTAA
DeltaAntigenF1_GenoA	1	GCCTCGAATAATAAGAAGAAAATTT
HBV Primers*		
HBV-F248	1&2	CTAGATTBGTGGTGGACTTCTCTCA
HBV-R397	2	GATARAACGCCGCAGATACATCCA
HBV-R450a	1	TCCAGGAGAACCAAYAAGAAAGTGA
HBV-R450b	1	TCCAGGAGAACCAAYAAGAAGATGA

*Primer sequences and PCR protocol described in (19)

Table S7.

Colony level demographic characteristics and PCR-based screening results of vampire bat blood and saliva for DrDV and HBV.

			DrDV-A		DrDV-B		HBV	
Colony	Prop Male*	Prop Adult†	Saliva	Blood	Saliva	Blood	Saliva	Blood
AYA1	0.6	1	0/20	0/20	3/20	0	0/3	0/20
AYA11	0.6	0.95	0/20	0/20	2/20	0	0/3	0/20
AYA14	0.4	0.65	1/20	0/20	4/20	0	0/8	0/20
AYA15	0.55	0.75	0/20	0	0/20	0	0	0
CAJ1	0.75	0.9	0/20	0	5/20	0/20	0/10	0/20
CAJ2	0.55	0.95	0/20	0	6/20	6/20	0/6	0/20
CAJ3	0.7	1	0/20	0	4/20	0	0/4	0
CAJ4	0.35	0.75	0/20	0	2/20	0	0/2	0
LMA4	0.65	0.75	0/20	0	0/20	0	0/1	0
LMA5	0.65	0.9	0/20	0	5/20	0	0/5	0
LMA6	0.35	1	0/20	0	7/20	4/20	0/9	0/19
LMA12	0.5	0.9	0/20	0	3/20	0	0/3	0
Total	-	-	1/240	0/60	41/240	10/60	0/54	0/119

*Proportion of males at each colony (alternative is females)

†Proportion of adults at each colony (alternatives are juveniles or subadults)

Table S8.

Test of association between DrDV-B phylogeny and sample location at the regional (department) and colony level.

Level	Index	Observed value (95% CI)	Null value (95% CI)	p-value
Region	AI*	0.22 (0-0.58)	2.46 (1.95-2.94)	0
	PS†	4 (3-5)	17.73 (15.41-19.35)	0
	MC‡ (LMA)	9.29 (5-14)	1.97 (1.41-2.98)	0.001
	MC‡ (CAJ)	11.24 (9-19)	2.81 (2.12-3.94)	0.001
	MC‡ (AYA)	2.67 (1-5)	1.26 (1-1.96)	0.02
Colony	AI*	2.23 (1.69-2.76)	3.63 (3.21-3.92)	0
	PS†	19.19 (18-20)	29.23 (27.45-30.81)	0
	MC‡ (LMA6)	4.98 (5-5)	1.18 (1-1.94)	0.001
	MC‡ (LMA5)	2.52 (1-3)	1.13 (1-1.43)	0.002
	MC‡ (CAJ2)	3.21 (2-6)	1.57 (1.14-2.39)	0.01
	MC‡ (CAJ1)	1 (1-1)	1.13 (1-1.53)	1
	MC‡ (AYA1)	1.09 (1-2)	1.01 (1-1.05)	1
	MC‡ (CAJ3)	1.01 (1-1)	1.07 (1-1.32)	1
	MC‡ (AYA11)	1.03 (1-1)	1.01 (1-1.05)	1
	MC‡ (AYA14)	1.05 (1-2)	1.04 (1-1.15)	1
	MC‡ (CAJ4)	1.16 (1-2)	1.01 (1-1.05)	1
	MC‡ (LMA12)	1 (1-1)	1.04 (1-1.15)	1

*Association Index
† Parsimony Score
‡ Monophyletic Clade size

Table S9.

Pooled bat saliva samples from Peru analyzed by metagenomic sequencing.

Genus	Species	Individuals in pool	Raw reads	Deltavirus contig length (bp)
Carollia	*perspicillata*	10*	28,700,978	N
Glossophaga	*soricina*	5	24,079,752	N
Desmodus	*rotundus*	10	28,946,275	921†
Diphylla	*ecaudata*	2	25,023,095	N
Anoura	*geoffroyi* *peruana*	6	18,569,505	N
Artibeus	*lituratus* *obscurus* *planirostris* *fraterculus*	10	14,966,399	N
Myotis	*oxyotus* *unidentified sp*	8	19,934,479	N
Sturnira	*erythromos* *unidentified sp*	6	11,348,995	N
Vampyressa/ *Vampyriscus*	*bidens* *unidentified sp*	4	13,734,389	N
Rare species	*Chiroderma trinitatum* *Chiroderma salvini* *Choeroniscus minor* *Rhynchonycteris naso* *Saccopteryx bilineata* *Messophyla macconelli* *Phyllostomus discolor* *Rhinophylla pumilio*	8	16,746,795	N

*Pool included individual CP-1 in which DrDV-B was detected by RT-PCR

†Pool was identical to CAJ1 pool where DrDV was initially discovered, confirming the ability to detect deltaviruses when they are known to be present

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