Diverse RNA viruses of arthropod origin in the blood of fruit bats suggest a link between bat and arthropod viromes

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Abstract

Bats host diverse viruses due to their unique ecology, behavior, and immunology. However, the role of other organisms with which bats interact in nature is understudied as a contributor to bat viral diversity. We discovered five viruses in the blood of fruit bats (*Hypsignathus monstrosus*) from the Republic of Congo. Of these five viruses, four have phylogenetic and genomic features suggesting an arthropod origin (a dicistrovirus, a nodavirus, and two tombus-like viruses), while the fifth (a hepadnavirus) is clearly of mammalian origin. We also report the parallel discovery of related tombus-like viruses in fig wasps and primitive crane flies from bat habitats, as well as high infection rates of bats with haemosporidian parasites (*Hepatocystis* sp.). These findings suggest transmission between arthropods and bats, perhaps through ingestion or hyperparasitism (viral infection of bat parasites). Some "bat-associated" viruses may be epidemiologically linked to bats through their ecological associations with invertebrates.

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1. Introduction

Many emerging viral pathogens have their origins in the fruit bats of the Old World (family *Pteropidae*). Pteropid bats are natural reservoir hosts of highly virulent pathogens such as Marburg virus, Nipah virus, and Hendra virus, and they are often presumed to host the ebolaviruses. Despite extensive studies of bats and their zoonotic or potentially zoonotic infections, little is knows about the broader ecology of bat viruses. To date, much emphasis has been placed on understanding direct spillover from bats to humans (Brierley et al., 2016; Marí Saéz et al., 2015; Plowright et al., 2015). Less effort has been placed on understanding the origins of viral diversity in bats or the maintenance of bat-associated viruses in nature. In particular, few studies have considered bat viruses in an ecological context that includes not only bats but also the diverse other taxa with which bats interact (Kuno and Chang, 2005; Malmlov et al., 2017).

Bats (order Chiroptera) are the second largest order of mammals after rodents (order Rodentia). Overall, bats host more emerging viruses per species than any other mammalian clade (Luis et al., 2013). The underlying ecological and phylogenetic diversity of bats contributes to the diversity and richness of viruses that they host (Luis et al., 2013; Webber et al., 2017; Willoughby et al., 2017). Other drivers of this viral diversity are varied (Luis et al., 2013), including factors related to viral exposure and persistence, such as long lifespans relative to body size (Munshi-South and Wilkinson, 2010), population connectivity (Plowright et al., 2011), the impacts of prolonged torpor (in some species) on immune function (Dempster et al., 1966), and diverse, densely aggregated multispecies roosting that can sustain chains of viral transmission (Willoughby et al., 2017).

Bats have close ecological relationships with arthropods (Allen et al., 1956; Goldberg et al., 2017; Kalka et al., 2008; Kuno and Chang, 2005; Palmer and Gunier, 1975; Sulkin et al., 1965). Most widely appreciated among these relationships is predation by insectivorous bats. Insectivorous bats consume insect biomass from 25% to greater than 100% of their body weight each night (Coutts et al., 1973; Kunz et al., 2011, 1995). Insectivorous bats are thereby likely exposed to a diversity of arthropod viruses through ingestion (Kuno and Chang, 2005), and a broad range of insect-origin viruses have been discovered in the guano of insectivorous bats (Kuno and Chang, 2005; Li et al., 2010; Reuter et al., 2014). These "dietary viruses" (Li et al., 2010; Reuter et al., 2014; Sulkin et al., 1965) result from passive transport through the gastrointestinal tract; however it is unknown whether viral nucleic acid or viral particles can cross the gut wall or infect bats in some cases.

Fruit bats, as their common name suggests, are dietary specialists on fruits. Fruit bats are among the most important seed dispersers in tropical forests, playing a critical role in forest regeneration and other ecosystem services (Kunz et al., 2011; Oleksy et al., 2017, 2015). Fruits, however, are themselves complex "ecosystems," with a diversity of closely associated

and often co-evolved arthropods in and on them. For example, one of the most specific fruit-arthropod associations is the obligate relationship between figs (family *Moraceae*, genus *Ficus*) and their highly specialized pollinating wasps (families *Agaonidae*, *Chalcidoidea*) (Yang et al., 2015). The "fruit" of the fig tree (syconium) is in fact the flower of the plant. The female wasp lays eggs in the syconium and dies. Her hatching male offspring tunnel through the "fruit" and die without exiting, while the female offspring exit through these tunnels, carrying pollen to another tree (Cook and West, 2005). Hammer-headed fruit bats (*Hypsignathus monstrosus)*, the focus of our study, consume figs as a primary food source, although their diet also includes the juice and pulp of mangoes, bananas, guavas, and soursops (Bradbury, 1977; Langevin and Barclay, 1990). Fruit bats therefore consume insects not merely incidentally, but rather as a significant component of their nutritional intake (Barclay et al., 2006; Clulow and Blundell, 2011; Herrera et al., 2002). Although opportunities for oral exposure of fruit bats to fruit-associated arthropod viruses are plentiful, this type of exposure is generally not considered in studies of viral ecology and evolution (Plowright et al., 2015).

Oral exposure of fruit bats to fruit-associated arthropod viruses is notably distinct from the transmission of vector-borne viruses by hematophagous arthropods. Vector-borne viruses transmitted by mosquitoes, including Chikungunya virus, West Nile virus, and Rift Valley fever virus have been detected in Pteropid bats (Boiro et al., 1987; Bunde et al., 2006; Diallo et al., 1999). Bat specialist ectoparasites such as bat flies (families **Nycteribiidae**, *Streblidae*) may also play a role as vectors (Dick and Dittmar, 2014). We recently described a novel rhabdovirus (family *Rhabdovirus*, genus *Ledantevirus*) of nycteribiid bat flies of fruit bats in Uganda with phylogenetic and genomic features that ally it with both zoonotic rhabdoviruses and insect-adapted viruses (Goldberg et al., 2017).

Here we describe a study of hammer-headed fruit bats in Republic of Congo in which we tested blood for viruses by next-generation sequencing (NGS). Our results reveal diverse viruses circulating in the blood of these fruit bats, four out of five of which are phylogenetically and genomically allied with viruses of arthropods. The new arthropod-origin viruses include a nodavirus (family *Nodaviridae*), a dicistrovirus (family *Dicistroviridae*), and two distinct variants of the recently recognized tombus-like viruses (family *Tombusviridae*). Furthermore, we surveyed the virome of *Ceratosolen* sp. fig wasps and primitive crane flies from bat habitats in Uganda and report novel tumbus-like viruses in these insects that are close relatives of the tombus-like virus from the blood of fruit bats, offering parallel evidence for the presence of such viruses in bat-associated arthropods. We also report high prevalence of a novel hepatitis B-like virus (Family: hepadnaviridae), a clearly mammalian virus, circulating in the same population of bats, as well as a high prevalence of infection with haemosporidian parasites of the genus *Hepatocystis*.

2. Results:

From 23 February 2015 to 1 March 2015, we captured 44 hammerheaded fruit bats (10 adult females, 29 adult males, 5 juvenile males) from a lekking site (described below). Analysis of NGS data from the serum of these bats revealed nucleic acid sequences from five previously unknown viruses, with near full genome coverage at sequencing depths of 531 reads per

base on average. Of the 44 bats sequenced, 1 (2.3%) contained sequences of a novel dicistrovirus (family *Dicistroviridae*), 1 (2.3%) contained sequences of and a novel nodavirus (family *Nodaviridae*), 3 (6.8%) contained sequences of one of two novel tombus-like viruses (family *Tombusviridae*), and 10 (22.7%) contained sequences of a hepatitis B-like virus (family *Hepadnaviridae*). Forty-two bats (95.5%) contained sequences of only one of these viruses, but 2 coinfected bats (4.5%) contained sequences from two of these viruses. Frequencies of infection with each virus are shown in Table 1.

2.1 Dicistrovirus

Dicistroviruses are dicistronic, picorna-like viruses, with two open reading frames (ORF) each accessed by independent internal ribosome entry sites (IRES). ORF1 encodes non-structural elements including a helicase, protease, and polymerase. ORF2 translation is mediated through the intergenic region (IGR) IRES and encodes the 4 capsid proteins (Nakashima and Uchiumi, 2009). The dicistrovirus we identified was of the genus *Cripavirus*. Cripaviruses are typically pathogenic in invertebrates, with the type strain, cricket paralysis virus (CrPV) causing paralysis and death in its natural host (Valles et al., 2017). Previously, a dicistrovirus was detected in insectivorous bat guano (Li et al., 2010; Reuter et al., 2014), presumably having passed through the gut.

1.9% of 728,482 trimmed reads from bat RML-1502254 mapped to what we provisionally named hypsignathus dicistrovirus, with deep coverage of both non-overlapping open reading frames (ORF1 and ORF2). Contiguous sequences (contigs) were assembled and trimmed reads were remapped iteratively to fill gaps and produce a viral consensus sequence of ~9300 nucleotides in length. The nucleotide identity of the capsid gene of the new virus to its closest known relative (drosophila C virus) is 73%. The species demarcation for dicistroviruses is accepted to be 90% identity in the capsid protein genes (Valles et al., 2017), making hypsignathus dicistrovirus a putative new species within the *Cripavirus* genus (Fig. 1A).

RT-PCR using hypsignathus dicistrovirus specific primers (ORF1) confirmed the presence of hypsignathus dicistrovirus in the sample identified as positive by NGS. RNA extract from water blanks were negative, confirming that the virus was not present as a contaminant of the silica columns. Retrospective analysis of samples and blanks run in our lab in the past showed no evidence of these viruses, further confirming that they did not result from contamination or cross-contamination.

2.2 Nodavirus

Nodavirus genomes are bipartite, with one segment coding for RNA replicase (RNA1: ~3.2 kb) and one segment (RNA2: ~1.2 kb) coding for the viral capsid precursor protein. Nodaviruses are separated into two genera, *Alphanodavirus* (arthropod infecting) and *Betanodavirus* (fish infecting). The alphanodaviruses, including the type strain Nodamura virus, have been detected in a range of insects including *Drosophila* fruit flies (Aguiar et al., 2015), hematophageous insects such as mosquitoes (Schuster et al., 2014; Tesh, 1980), and phlebotomine sandflies (Aguiar et al., 2015).

We obtained ~2kb of the RNA replicase of segment RNA1 of a novel alphanodavirus. The sequence was most closely related to the type strain, Nodamura virus (Johnson et al., 2004)., and clusters with Nodamura virus phylogenetically (Fig. 1B), but with a percent amino acid identity of only 54%. For convenience, we refer to this virus as hypnovirus ("hypsignathus nodavirus"), indicating its discovery in *H. monstrosus* and its being a putative new genus (*Hypnovirus*, provisionally), following naming conventions for nodaviruses discovered in the feces of canines (*Caninovirus*) (Conceição-Neto et al., 2017) and in mosquitos (*Mosinovirus*) (Schuster et al., 2014). The next most closely related nodavirus, lutzomyia nodavirus, infects phlebotamine sandflies and is considered potentially vector-borne (Aguiar et al., 2015).

2.3   Tombus-like viruses

Tombus viruses, named for the type strain tomato bushy stunt virus, are positive sense, single stranded RNA viruses that typically infect plants (Stuart et al., 2004). However, recently discovered tombus-like viruses have diverse hosts, including marine invertebrates, terrestrial arthropods, and potentially free-living or parasitic protists (Dolja and Koonin, 2018; Shi et al., 2016). Tombus-like viruses have diverse genome architectures, utilizing both segmented and non-segmented strategies (Shi et al., 2016).

We detected RNA-dependent RNA polymerase (RDRP) sequences of two distinct clades of tombus-like viruses in *H. monstrosus* (Fig. 1C). The first, tentatively named hypsignathus monstrosus tombus-like virus 1, most closely resembled Wuhan insect virus 35 (2221 bp genome with two overlapping ORFs), a tombus-like virus discovered in an insect pool from Wuhan, China (Shi et al., 2016), but was highly divergent (54% amino acid identity). In two other *H. monstrosus*, we detected similar sequences (96% nucleotide identity) representing a second tombus-like virus, tentatively named hypsignathus monstrosus tombus-like virus 2.Hypsignathus monstrosus tombus-like virus 2 was a ~90% complete genome (3600 bp), and by BlastX (Altschul et al., 1990) was most closely related by to Beihai tombus-like virus 7 (3939 bp genome with 3 ORFS, 2 overlapping), originally detected in panaeid shrimp from Beihai, China. However amino acid percent identity was only 39% between hypsignathus monstrosus tombuslike virus 2 and Beihai tombus-like virus 7.

In 1 pool of 20 *Ceratosolen sp.* fig wasps (order Hymenoptera, family *Agaonidae*) collected from a *Ficus brachylepis* syconium and in 3 pools of two primitive crane flies each (order Diptera, family *Tanyderidae*) from fruit bat habitats in Uganda (hollow roosting trees of the genera and species *Pterygota mildbraedii*, *Olea witchii*, and *Parinari excelsa*), we also detected tombus-like virus sequences representing partial RDRP genes. These viruses cluster with Wuhan insect virus 35 and hypsignathus monstrosus tombus-like virus 1. In particular, fig wasp tombus-like virus shared 52.5% identity with hypsignathus monstrosus tombus-like virus 1, and the primitive crane fly tombus-like virus shared 60% identity with hypsignathus monstrosus tombus-like virus 1, making crane fly tombus-like virus the closest known relative of hypsignathus monstrosus tombus-like virus 1.

2.4 Hepadnaviruses

22.7% of *H. monstrosus* sampled (10/44) contained a novel hepatitis B-like virus provisionally named hypsignathus monstrosus hepatitis B virus (HMHBV). Phylogenetic analysis based on ~1000 nt of the hepatitis B polymerase gene (pol) indicates that HMHBV clusters with rodent orthohepadnaviruses, sharing 72% nucleotide identity with Woodchuck Hepatitis virus (Fig. 1D). In contrast, HMHBV pol shares only 50% nucleotide identity with the nearest bat hepadnavirus, tent-making bat hepatitis virus (Drexler et al., 2013).

2.5 Haemosporidian parasites

In addition to the viruses described above, we detected small subunit ribosomal RNA gene sequences of the haemosporidian parasite genus *Hepatocystis* in 43/44 (97.7%) bats (GenBank accession numbers MK234751-MK234793). These sequences were very similar to each other on average (98.5% ± 0.3% SE nucleotide identity) and were most closely related (mean 92.3% ± 1.2% SE nucleotide identity) to *Hepatocystis sp.* isolate CRNP11 (GenBank accession number DQ396536) detected in a Horsfield's fruit bat (*Cynopterus horsfieldii*) in Malaysia. Of the 43 *Hepatocystis*-infected bats, 3 were also infected with hypsignathus monstrosus tombus-like viruses (1 and 2), 1 with hypnovirus and 1 with hypsignathus dicistrovirus.

2.6 Analysis of dinucleotide frequencies

In order to explore virus-host associations, we calculated dinucleotide frequencies of the novel viral sequences we discovered. The new tombus-noda viruses detected in *H. monstrosus* do not exhibit a depletion of CpG dinucleotides nor an excess of dinucleotide TpA, as is typical of mammalian and plant RNA viruses (CpG: Avg 0.94 StDev: 0.09; TpA: Avg 0.82, StDev 0.23) (Glass et al., 2007; Upadhyay et al., 2013). Rather, values for both CpG and TpA of arthropod tombus-like viruses were statistically indistinguishable from Tombus-Noda viruses identified in bats and other mammals (CpG: Two-Tailed P-Value 0.86; TpA: 0.5752). Hypsignathus dicistrovirus showed limited CpG depletion and no excess of TpA (CpG: 0.67, TpA: 0.77), with both values falling within have a standard deviation of average for dicistroviruses (Average CpG: 0.75, StDev: 0.15; Average TpA: 0.78, StDev: 0.08). To compare these values directly, we conducted two principal components analyses of dinucleotide frequencies of the Picornavirales (the picornaviruses and dicistroviruses) and the Tombus-Noda clade of viruses (Fig. 2). This analysis shows that variations in dinucleotide frequencies can discriminate between related viruses of vertebrates and invertebrates, as well those of invertebrates and plants. In both analyses, the viruses we detected in *H. monstrosus* cluster with viruses of arthropods.

3. Discussion

Many bats are "keystone" species, in that their absence would have cascading effects across ecosystems (Kunz et al., 2011). This high ecological connectivity may predispose bats to exposure to viruses of the species with which they interact in nature, including a diversity of arthropod viruses. Our results suggest that, in at least some cases, arthropod viruses can be found in bat blood. Although bites by vectors are a well-established route by which arthropods transmit viruses to bats (Klimpel and Mehlhom, 2013), our results suggest that

other mechanisms may also be important. Oral exposure may be one such mechanism. "Fruit" bats (despite their common name) consume a great biomass and diversity of arthropods on fruits, not simply incidentally but as an important nutritional component of their diets (Barclay et al., 2006; Clulow and Blundell, 2011; Herrera et al., 2002). Our discoveries of diverse, novel, arthropod origin viruses in the blood of frugivorous bats suggests that such interactions might create an "ecological link" between arthropod and bat viromes.

Dicistroviruses have been identified in the feces of mammals, including insectivorous bats and even humans, but the fecal virome includes food-associated viruses that may simply have passed through the gut (Janowski et al., 2017; Kapoor et al., 2010; Reuter et al., 2014). Viruses of plants or insects detected in feces are frequently present due to dietary exposures, but may not indicate replicative infection (Balique et al., 2015; Zhang et al., 2006). It remains unclear whether arthropod dicistroviruses can replicate in mammalian cells. Conflicting reports have been published regarding the culture of taura syndrome virus in primate cell lines (Audelo-del-Valle et al., 2003; Pantoja et al., 2004). Our finding of a dicistrovirus circulating in the blood of a fruit bat could indicate passage of viral material from the gastrointestinal to the circulatory system. We speculate that such a process might occur through micro-abrasions during mastication and swallowing of rough food matter (Soave, 1966). Given its high level of similarity to drosophila C virus, hypsignathus dicistrovirus may have been acquired through ingestion of a dipteran insect associated with fruit.

The diversity of tombusviruses, nodaviruses, and the intermediary "tombus-like" viruses such as those in the blood of hammer-headed fruit bats has only recently become elucidated (Shi et al., 2016). Tombus-noda viruses are a super-family of single stranded positive sense RNA viruses with diverse hosts (Longdon et al., 2014). Tombus-noda virus clades contain phylogenetic admixtures of viruses of nematodes, marine invertebrates, terrestrial invertebrates, plants and mammals. Host-switches have been common over evolutionary time, although the current cross-species transmission potential of tombus-noda viruses requires further study (Ball et al., 1992; Johnson et al., 2004; Tesh, 1980).

Our parallel discoveries of tombus-like viruses in fig wasps and primitive crane flies associated with bat habitats strengthens the argument for a link between bat and arthropod viromes. Hypsignathus monstrosus tombus-like virus 1 is most closely related to the new tombus-like virus from primitive crane flies, which are commonly associated with habitats frequented by forest roosting bats. Also closely related to the tombus-like viruses of hammer-headed fruit bats are the new viruses from fig wasps of *F. brachylepis* figs. These wasps have evolved mutualistic relationships with fig trees, laying eggs inside figs and serving as their pollinators (Wang et al., 2010). Figs are important foods for many frugivorous animals across the tropics, including bats (Shanahan et al., 2001). Hypsignathus monstrosus tombus-like virus 2 falls within a clade with Beihai tombus-like virus 8 and other marine invertebrate-hosted tombus-like viruses, suggesting the existence of unknown tombus-like viruses in invertebrates of terrestrial origin with which fruit bats interact.

Alternatively, such viruses may be hosted by bat ecto- or endoparasites and thus be "hyperparasites" (Dolja and Koonin, 2018; Goldberg et al., 2017; Grybchuk et al., 2018; Jansen van Vuren et al., 2017, 2016). For example, a recent study detected fungus-associated partitiviruses in human sera and on that basis inferred viral hyperparasitism of a fungal pathogen (Phan et al., 2018). In this light, we note the high prevalence of infection of the sampled bats with haemosporidian parasites of the genus *Hepatocystis*, which are common in African fruit bats (Perkins and Schaer, 2016; Schaer et al., 2017) The tombus-like viruses we detected could be viruses of this or another parasite. Such hierarchical ecological associations complicate traditional definitiions of "host" but recall the notion of the "holobiont" virome, which considers the assemblage of a host and its parasites as an ecological unit (Richardson 2017).

By contrast, hypnovirus, the novel nodavirus we detected in hammer-headed fruit bat sera, is likely vector-borne. The type strain, Nodamura virus, was first detected in a mosquito, but nodaviruses have been shown to cause lethal infections in insects, mammals, and fish (Bailey and Scott, 1973; Shetty et al., 2012; Tesh, 1980). Transfection of Nodamura virus genomic RNAs has resulted in replication in a wide variety of cultured cells, showing intracellular competence for cross species transmission (Ball et al., 1992). The close evolutionary relationships of hypnovirus to the vector-borne alphanodavirus Nodamura virus and the more recently discovered phlebotomine sandfly lutzomyia nodavirus (Aguiar et al., 2015) suggests that this virus was detected at a time of transient viremia following transmission by a hematophagous arthropod. Sampling of potential vectors at the lekking site would be necessary to identify the vector.

Our detection of a novel hepatitis B virus, hypsignathus monstrosus hepatitis B virus (HMHBV), re-enforces the notion that our data accurately reflect the true blood virome of this bat population. Hepadnaviruses are small, partially double-stranded DNA viruses with circular genomes that infect a diversity of mammals, and the closest relatives of HMHBV are hosted by ground squirrels (Testut et al., 1996) and woodchucks (Tyler et al., 1981). Rodent hepadnaviruses have been discovered from the Arctic to the temperate regions, and bat hepadnaviruses have been detected in the Americas, Asia, and West African insectivorous bats (Drexler et al., 2013; Rasche et al., 2016; Wang et al., 2017), but not previously, to our knowledge, in African pteropids. HMHBV shared the greatest nucleotide identity (72%) in the polymerase gene with woodchuck hepatitis virus, and was only distantly related to the most similar known bat hepatitis B virus (Drexler et al., 2013). This places HMHBV in what was previously considered the rodent clade of hepatitis B viruses (Testut et al., 1996).

Dinucleotide frequency analysis offers further insights into the patterns observed above. Dinucleotide frequency can offer insight into viral host adaptation, at least across widely divergent host biologies (e.g. vertebrates versus invertebrates, and plants versus invertebrates; Babayan et al., 2018; Kapoor et al., 2010; Upadhyay et al., 2014). RNA viruses of vertebrates and plants typically show a depletion of CpG dinucleotide, and an excess of TpA dinucleotide. By contrast, RNA viruses of invertebrates do not exhibit extreme CpG and TpA frequencies (Kapoor et al., 2010; Karlin et al., 1994; Rima and McFerran, 1997). The CpG and TpA frequencies of the Tombus-Noda viruses and

dicistroviruses that we detected in hammer-headed fruit bats were statistically indistinguishable from frequencies in related viruses adapted to the cellular replication machinery of arthropods.

Our results bear on a long-standing problem in infectious disease ecology. Some viruses that are considered "bat-associated" have not yet been isolated from bats. Most famous among these are the ebolaviruses which have yet to be isolated from bats (Leendertz et al., 2016) but have been detected in bats by PCR (Goldstein et al., 2018; Leroy et al., 2005) and antibody tests (Hayman et al., 2012, 2010; Ogawa et al., 2015; Pourrut et al., 2009, 2007). Over evolutionary time, many medically important viral taxa appear to have originated in invertebrates, with viruses of mammals in general, and bats in particular, interspersed among those of arthropod hosts (Shi et al., 2016). Our results provide a mechanism by which such a pattern might occur – namely frequent transmission of invertebrate viruses to bats via non-vector-borne modes of transmission such as ingestion or hyperparasitism.

For example, we recently documented an unusual rhabdovirus in nycteribiid bat flies (obligate hematophagous ectoparasites of bats) on pteropids in Uganda (Goldberg et al., 2017). This virus is a member of the genus *Ledantevirus*, which contains the zoonotic Le Dantec virus (Woodruff et al., 1977). Members of this viral genus are considered "bat associated" despite some never having been found in bats themselves (Blasdell et al., 2015; Goldberg et al., 2017). Bats may be associated ecologically and evolutionarily with these "bat associated" viruses not as reservoirs, but rather as intermediary hosts of arthropod viruses. The discovery of Bombali ebolavirus in Sierra Leone validates the consensus view that bats play a role in the ecology of ebolaviruses, but because it was found in two sympatric insectivorous bat species the exact nature of its association with bats remains unclear (Goldstein et al., 2018). Since the initial discovery of Sudan ebolavirus, it has been speculated based on circumstantial evidence that the reservoirs of certain ebolaviruses might non-hematophagous arthropods (Leendertz, 2016; Monath, 1999; Preston, 2012). Our results support the plausibility of this notion and suggest that occasional arthropod-bat transmission under ecologically favorable conditions might account for the sporadic appearance of such "bat associated" viruses.

Virus discoveries in apparently discordant hosts and sample types must be carefully scrutinized. In rare cases such discoveries have been linked to contaminated laboratory reagents (Naccache et al., 2013; Simmons et al., 2011). For example, marine-sourced silica in nucleic acid extraction columns has previously been linked to the unlikely presence of a marine hybrid parvovirus-like virus in samples from human patients (Naccache et al., 2013). Although marine dicistroviruses exist (Bonning and Miller, 2010), hypsignathus dicistrovirus is phylogenetically allied with terrestrial dicistroviruses (and absent from other samples analyzed previously, concurrently, and subsequently in our lab). Furthermore we were able to detect hysignathus dicistrovirus by NGS and RT-PCR amplification only in some bat serum samples and not in other bat serum samples or in extraction blanks. Hypsignathus dicistrovirus is not therefore a misassigned marine dicistrovirus contaminant of the silica columns, but rather a virus in the blood of the bats.

We also caution that our inferences are based on the detection of viral nucleic acid in the serum of bats, and not on detection of live viruses. Although complete genes of naked, dietary double-stranded DNA can pass from gut into the serum (Spisák et al., 2013), the half-life of unprotected viral RNA is significantly shorter (Chen et al., 2008; Dickson and Wilusz, 2011). Unfortunately, biosafety considerations required inactivation of all samples prior to processing. Nevertheless, we do not believe the viruses we detected are incidental. The coverage and depth of sequencing varied across the viruses discovered, ranging from 80-100% genome coverage, and up to 500x average coverage depth. Canonical ORFs did not contain premature stop codons. Bat and insect samples were sequenced on different days, and we have never before sequenced viruses similar to those reported herein.

Overall, our discovery of diverse arthropod origin viruses circulating in the blood of hammer-headed fruit bats of the Republic of Congo, as well as our discovery of a new mammalian virus related to rodent orthohepadnaviruses, are suggestive of diverse modes of viral transmission, all of which may all contribute to the observed diversity of viruses in bats. We suspect that cross-species transmission of viruses to bats and other mammals from invertebrates may occur with more regularity than has been appreciated, and that arthropods may host many "bat associated" viruses that have defied detection in bats themselves. Further analysis of these and other samples of arthropods from bat habitats is warranted. Future efforts to explore alternative reservoirs of "bat associated" viruses should include sampling of invertebrates that are ecologically associated with bats and that may host viruses capable of infecting bats.

4. Methods:

4.1 Collection of samples

The study site lies in the buffer corridor of Odzala-Kokoua National Park (OKNP), along National Route 2 roughly 100 km southwest of Ouesso in the Republic of the Congo (00° 54' 33" N and 15° 36' 01" E). Hammer-headed fruit bats (*Hypsignathus monstrosus*) were captured at night using canopy-level mist nets at a lekking site and transported to the sampling set-up in cloth bags. After sampling, the bats were released at the location of capture. Blood samples were obtained from the cephalic vein and centrifuged in MiniCollect Z serum tubes (Greiner Bio-One). Sera was collected and frozen in liquid nitrogen in the field and stored at −80°C in Brazzaville, Republic of the Congo, until shipment to the USA. All methods approved by Institutional Animal Care and Use Committee of the Rocky Mountain Laboratories (NIH ASP #2015-010).

In a related effort, in January 2016, 80 samples of fig wasps (4 pools) from 4 species of figs (*F. brachylepis*, *F. spongii*, *F. mucuso*, and *F. capensis*) were collected from locations in and near Kibale and Semliki National Parks, in western Uganda. Briefly, ripe, intact synconia were collected from the ground underneath the trees and were opened using sterile instruments. Adult fig wasps and larvae (encased in galls) were collected into sterile tubes containing DNA/RNA Shield buffer (Zymo Research Corporation, Irvine, CA, USA). Primitive crane flies were collected from 4 hollow tree bat roosts (*Pterygota mildbraedii*, *Olea witchii*, *Parinari excelsa*, *Strombosia scheffleri*) using sterile forceps and also placed

into sterile tubes containing DNA/RNA Shield buffer. Insect samples were then processed using previously described methods (Goldberg et al., 2017).

4.2 Virus detection and characterization

RNA was extracted from samples (200-300 uL of bat serum or homogenized insects), using QIAamp MinElute Virus Spin kit (Qiagen Inc., Valencia, CA), without carrier RNA and stored at –80°C until shipment on dry ice to the University of Wisconsin-Madison for NGS. NGS was performed using methods previously described (Bennett et al., 2016; Toohey-Kurth et al., 2017). Briefly, RNA was DNAse treated by using the Turbo DNA-free Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Extracted, DNAse treated, RNA was then converted to double-stranded cDNA (dscDNA) using the Superscript double-stranded cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) with random hexamer priming of first-strand synthesis. Double stranded cDNA was purified using Agencourt Ampure XP beads (Beckman Coulter, Brea, CA, USA). DscDNA was prepared for paired-end NGS by Illumina MiSeq (MiSeq Reagent Kit v3, 300 cycle, Illumina, San Diego, CA, USA) using the Nextera XT DNA sample prep kit (Illumina, San Diego, CA, USA). NGS reads were analyzed as previously described (Goldberg et al., 2017).

4.3 Nucleotide composition analyses

The relative frequency of CpG dinucleotide pairs was calculated for CDS of each virus consensus sequence using the R Biostrings package (Pages et al., 2017). Relative dinucleotide frequency is the ratio of the observed to the expected frequency of a particular dinucleotide. A dinucleotide is considered underrepresented when the relative dinucleotide frequency is <0.78, and overrepresented when >1.22. Statistical analyses were performed in the computer package R (R Core Team, 2013). Principal components analysis of relative dinucleotide frequencies of all 16 dinucleotides was performed in R using the Stats package.

4.4 Phylogenetics

Maximum likelihood phylogenetic analyses were performed with codon-based alignments of polymerase genes. Alignments were created using the MAFFT algorithm (Katoh et al., 2002) in TranslatorX (Abascal et al., 2010), using Gblocks (Talavera et al., 2007) to exclude poorly aligned regions. Maximum likelihood phylogenies were constructed in PhyML (Guindon et al., 2010) and displayed utilizing FigTree (Rambaut, Andrew, 2016).

4.5 Quality control RT-PCR

RT-PCR primers were developed for hypsignathus dicistrovirus ORF1 (HDorf1F: 5' – TTG CAG CAA AAC AGT TGA GG – 3'; HDorflR: 5' - TGA GAC CAC AAA CCC AGA CA – 3') to confirm NGS-based results and to test laboratory reagents as a potential source of contamination. RNA from NGS positive sera and water blank negative controls were extracted using both trizol and Qiagen column based methods (QIAamp MinElute Virus Spin kit; Qiagen Inc., Valencia, CA). RT-PCR was performed using New England Biolabs OneTaq One-Step RT-PCR kit (New England Biolabs, Ipswich, Mass.) under standard conditions, with the following thermocycling parameters: 48°C for 30 min; 94°C for 60 sec; 40 cycles at 94°C for 15 sec, 51°C for 30 sec, 68°C for 45 sec; 68°C for 5 min. PCR

products were visualized under ultraviolet light on 1.5% agarose gels stained with ethidium bromide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

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Highlights:

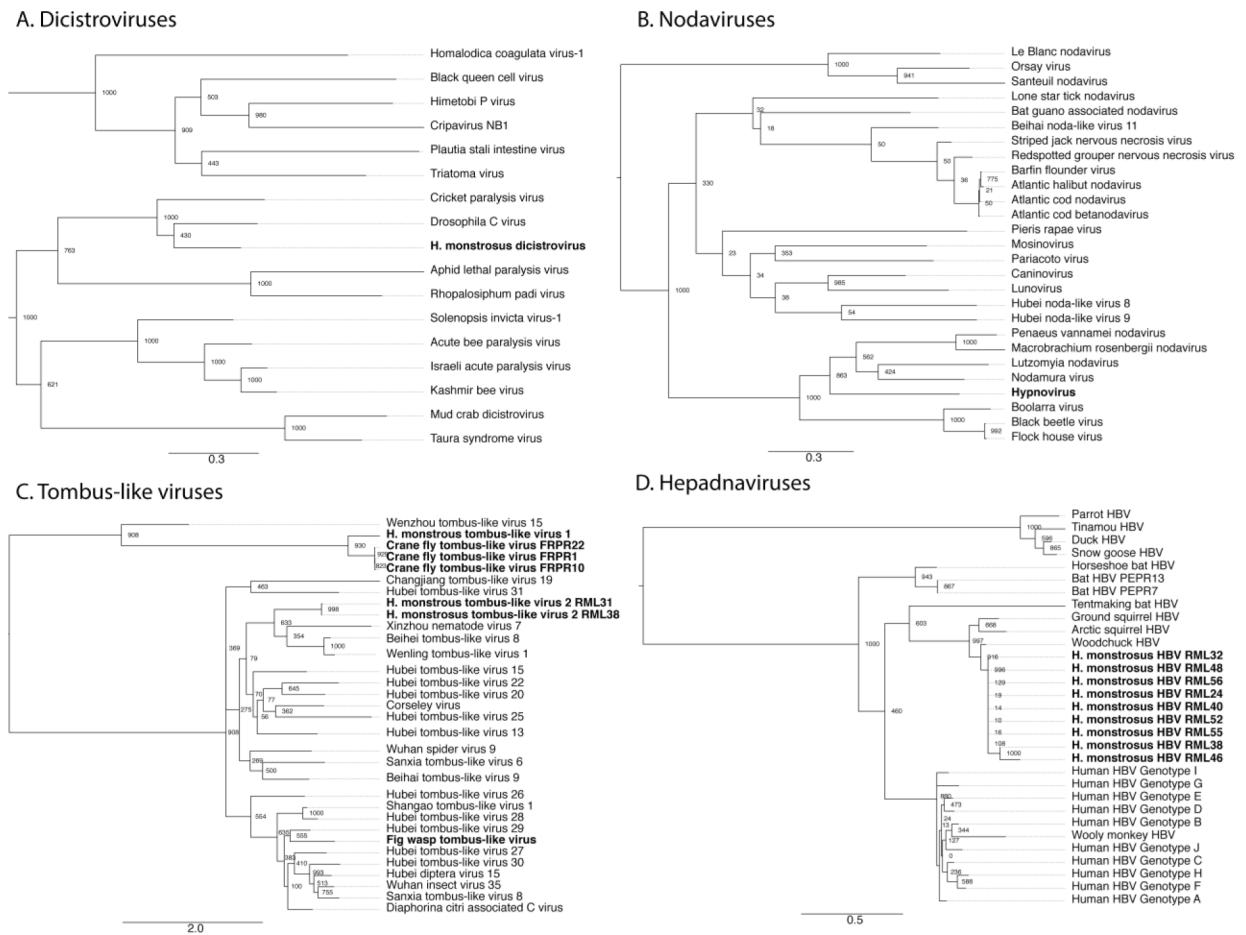- Fruit bats from Republic of Congo host diverse RNA viruses of arthropod origin.

- Four arthropod viruses were found in the blood of bats at a forest breeding site.

- Bats may acquire invertebrate viruses through their diets or from bat parasites.

- "Bat associated" viruses may reside in invertebrates with ecological links to bats.

Figure 1.
Maximum likelihood phylogenies (1,000 bootstrap replicates) of polymerase genes sequences of **A.** dicistroviruses (~2700 bp), **B.** nodaviruses (~2000 bp), **C.** tombus-like viruses (~1200 bp), and **D.** hepadnaviruses (~570 bp). Maximum likelihood phylogenetic analyses were performed with codon-based alignments of polymerase genes, with poorly aligned regions removed (see text for full description).
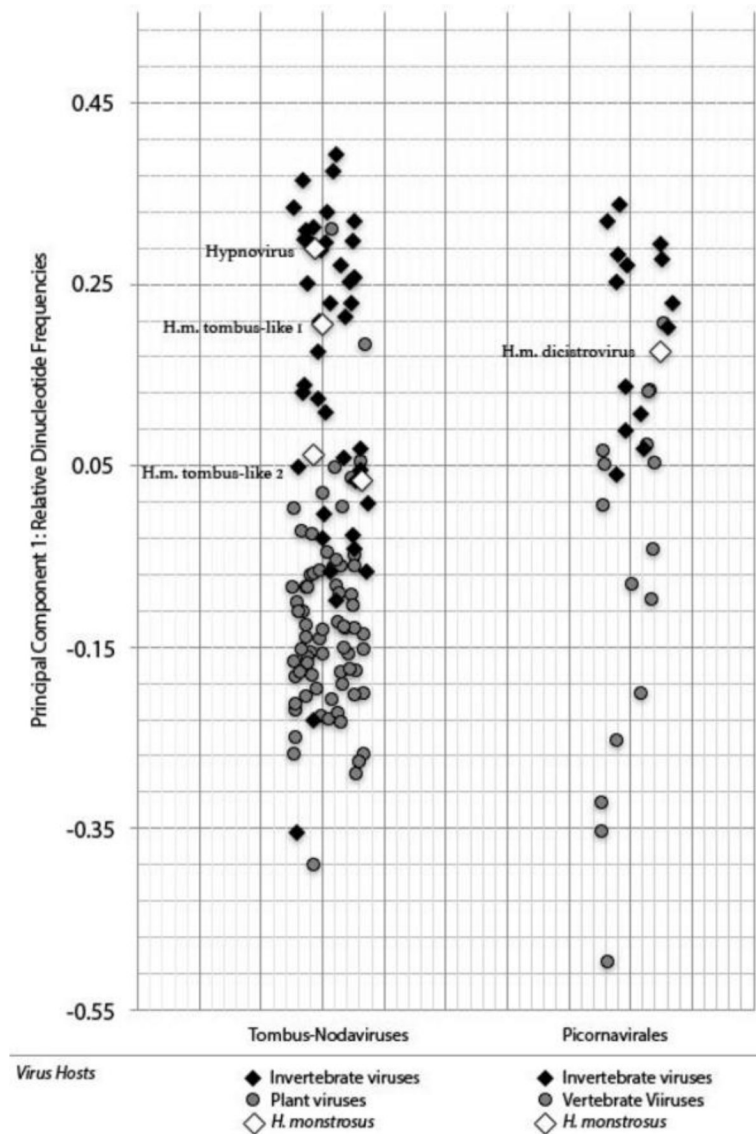
Figure 2.
Principal components analysis of relative dinucleotide frequencies of Tombus-Noda viruses
and Picornavirus-dicistroviruses, from a diversity of hosts. The plot shows separation of
groups by the first principal component, which can be interpreted as the direction,
uncorrelated to other components, which maximizes the variance of the samples when
projected onto the component. Points represent individual sequences.

Table 1:

Novel viruses in Hammerhead fruit bats

	Arthropod Origin				Vertebrate Origin
	Dicistrovirus	Nodavirus	Tombus-like virus		Hepadnavirus
closest relative:	Drosophila C Virus	Nodamura Virus RNA 1	Wuhan Insect Virus 35	Beihai tombus-like virus 7	Woodchuck Hepatitis Virus
Accession	MH310078	MH324435	MH324433	MH324432	MH324435
Percent identity	73%	54%	69%	39%	72%
Genome Coverage	80%	74%	100%	90%	20%
Prevalence (n=44)	2.3%	2.3%	2.3%	4.5%	22.7%
CpG Frequency	0.7	1.0	0.9	0.8	0.7