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Zoonotic disease and virome diversity in bats

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The emergence of zoonotic viral diseases in humans commonly reflects exposure to mammalian wildlife. Bats (order Chiroptera) are arguably the most important mammalian reservoir for zoonotic viruses, with notable examples including Severe Acute Respiratory Syndrome coronaviruses 1 and 2, Middle East Respiratory Syndrome coronavirus, henipaviruses and lyssaviruses. Herein, we outline our current knowledge on the diversity of bat viromes, particularly through the lens of metagenomic next-generation sequencing and in the context of disease emergence. A key conclusion is that although bats harbour abundant virus diversity, the vast majority of bat viruses have not emerged to cause disease in new hosts such that bats are better regarded as critical but endangered components of global ecosystems.

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Introduction

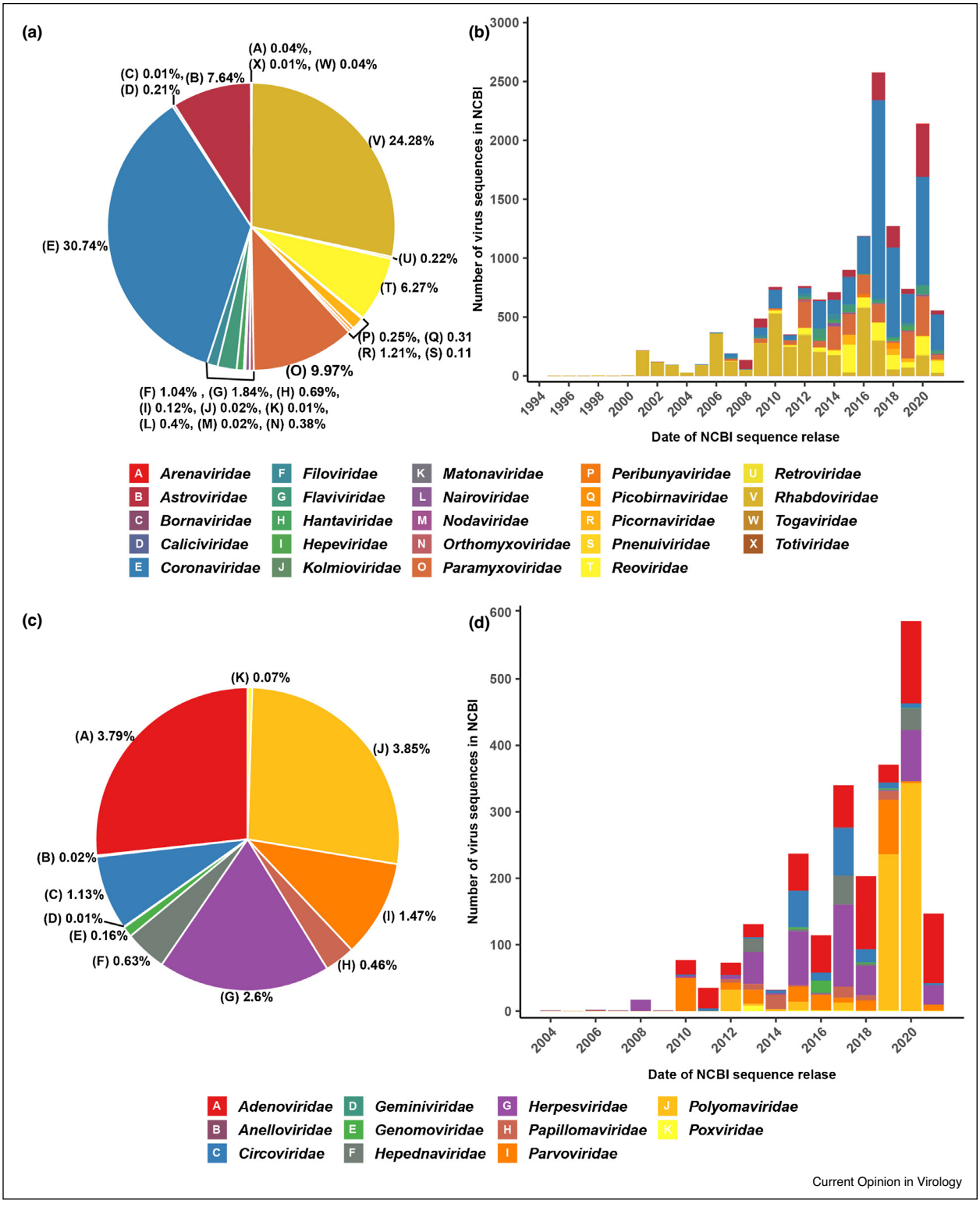
The global COVID-19 pandemic caused by Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) has intensified interest in bats as reservoirs for emerging viruses. Bats are the largest mammalian order (Chiroptera) after rodents, comprising over 1400 species from two suborders: the Yinpterochiroptera containing the *Pteropodidae* ('fruit bats') and five families of microbat, and the Yangochiroptera containing the remaining 14 microbat families [1•]. The most widespread bat families include the *Hipposideridae*, *Pteropodidae*, *Rhinolophidae*, *Molossidae*, *Emballonuridae*, *Phyllostomidae* and *Vespertilionidae* that contain up to ~190 species, while the *Craseonycteridae* and *Myzopodidae* harbour only one or two species and are geographically restricted [2,3]. For dietary purposes bats can be classified as insectivores, frugivores, carnivores and nectivores, and several species use echolocation to characterise their surrounding environment and locate prey. Bats are found in every continent excluding Antarctica, in part reflecting their capacity to migrate via flight, with roosting sites that vary seasonally.

Virome diversity in bats

Metagenomic sequencing is an increasingly powerful and popular tool for virus discovery, particularly as it allows the characterisation of viruses from families that are often overlooked in PCR-based screening that focuses on known or likely pathogens. It is therefore no surprise that much of our knowledge of bat viromes comes from the deployment of large-scale metagenomic next-generation sequencing (mNGS), particularly total RNA sequencing (metatranscriptomics). At the time of writing, ~30% of all the bat associated virus sequences deposited on NCBI/GenBank have been identified by mNGS only, an increase from 10% in 2016 (Figure 1). The recent identification of Wittenau bat nairovirus (*Nairoviridae*) and Ruhugu virus (*Matonaviridae*) serve as informative examples [4,5], as was the detection of deltaviruses (*Kolmioviridae*) in bats, even though these viruses were originally only associated with humans in the context of co-infection with hepatitis B virus [6•].

The collection of bat urine, saliva and faecal samples is commonly used in mNGS studies as this minimises the impact on bat populations. In many cases it also represents the likely route of virus transmission, although it may miss viruses associated with specific tissues. The analyses of faecal material and urine from bat species sampled on multiple continents have identified viruses from the *Adenoviridae*, *Astroviridae*, *Caliciviridae*, *Coronaviridae*, *Flaviviridae*, *Papillomaviridae*, *Paramyxoviridae*, *Parvoviridae*, *Picornaviridae*, *Polyomaviridae* and *Reoviridae* [7,8•,9–11]. For example, a study of more than 4000 bat rectal and pharyngeal swabs from three common bat genera – horseshoe bats (*Rhinolophus*), mouse-eared bats (*Myotis*) and bent winged bats (*Miniopterus*) – in China identified novel virus sequences from diverse viral families, including the *Coronaviridae* and *Paramyxoviridae* (see below), as well as those from the order *Bunyavirales* of RNA viruses [12••]. The *Bunyavirales* currently comprises 12 families including a number associated with human disease. Five of these families have been identified in bats: the *Arenaviridae*, *Nairoviridae*, *Hantaviridae*, *Peribunyaviridae* and *Phenuiviridae* [5,8•,13,14]. However, it is also important to note that rather than infecting bat themselves, many of the viruses detected in bats may be associated with aspects of the bat diet and microbiome. This is especially the case in studies utilising faecal

Figure 1



Taxonomic distribution of publicly available gene sequences of bat viruses. Plots show virus sequences on NCBI/GenBank in which the order Chiroptera or individual bat species are listed as hosts. **(a)** Percentage of bat virus sequences that belong to RNA virus families and **(b)** RNA virus sequences by year of NCBI release. **(c)** Percentage of bat virus sequences that belong to DNA virus families and (D) DNA virus sequences by year of NCBI release.

mNGS studies [8•,9,15–17], and care should also be taken to exclude reagent contamination [18].

A broad-scale conclusion from metagenomic studies is that bats may be particularly prone to carrying viral families that are commonly associated with zoonotic disease. Of the more than 16 600 bat associated viral sequences on NCBI/GenBank, 85% are RNA viruses, including 30% and 24% from the families *Coronaviridae* and *Rhabdoviridae*, respectively, while 10% of all bat-associated viral sequences identified to date are from the *Paramyxoviridae* (Figure 1). Interestingly, those virus families that might pose a greater zoonotic risk (i.e. the *Coronaviridae*, *Paramyxoviridae* and *Rhabdoviridae*; see below) are generally associated with a narrower range of bat families compared to those viruses that may be less likely to emerge in humans (i.e. *Astroviridae*, *Reoviridae* and *Picornaviridae*) (Figure 2). As a case in point, a range of SARS-CoV-2 related coronaviruses have been characterised through mNGS of bats sampled in China and parts of south-east Asia [19••,20,21]. The viruses carried by bats also vary markedly by bat family (Figure 2). As expected, the widespread and species rich bat families (i.e. the *Vespertilionidae*, *Rhinolophidae*, *Pteropodidae*, *Phyllostomidae* and *Hipposideridae*) harbour a greater diversity of viruses than the less speciose families (*Noctilionidae*, *Natalidae*, *Myzopodidae* and *Furipteridae*).

Notable zoonotic outbreaks associated with bats

Coronaviridae

The current interest in bats as reservoirs for emerging viruses began with the outbreak of SARS-CoV-1 in 2002/2003 [22], and there has recently been intense research activity in documenting the diversity of alphacoronaviruses and betacoronaviruses (Figure 3).

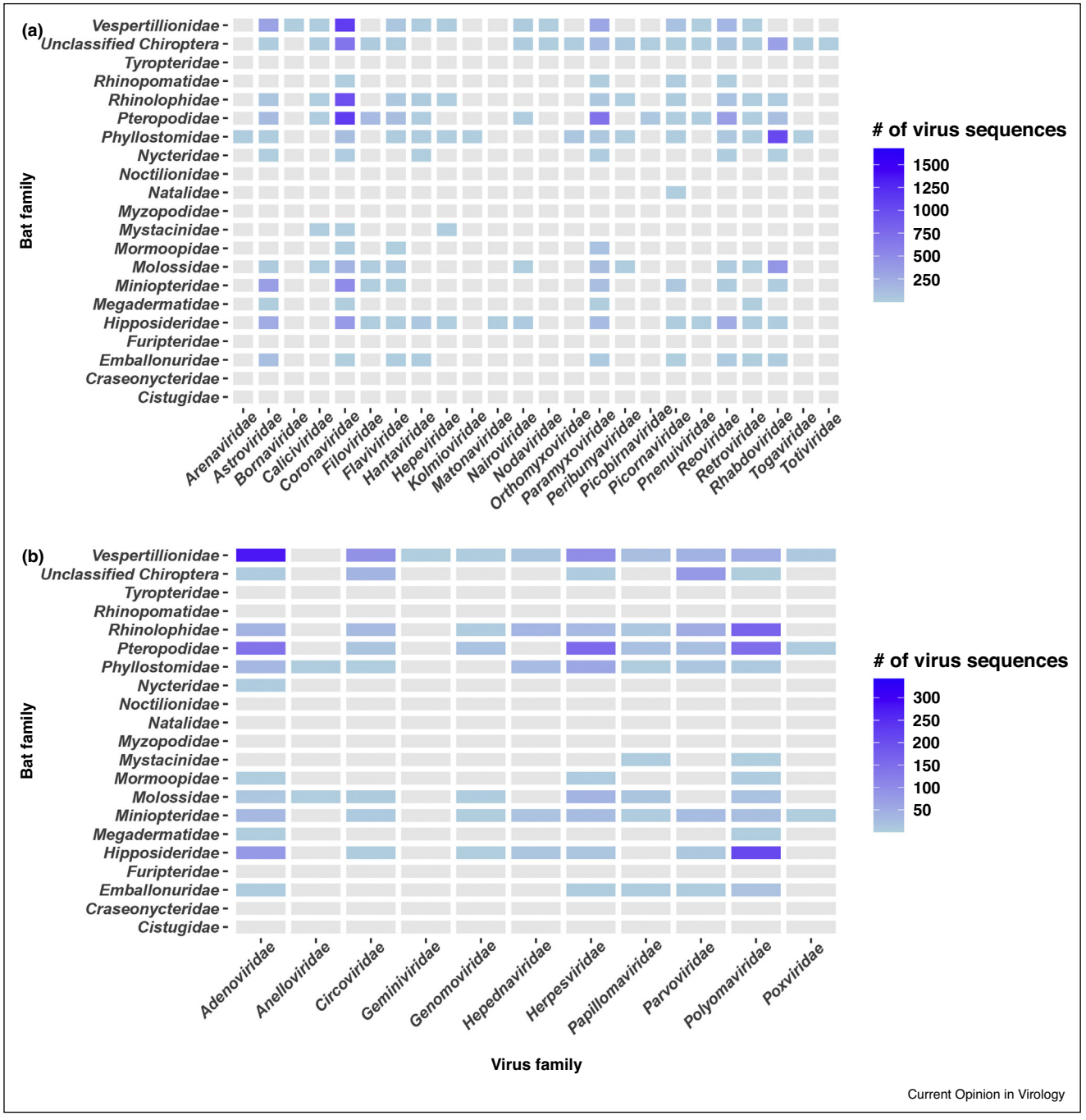
Initial studies showed that civets (*Paguma larvata*) were the likely source of SARS-CoV-1, with emergence in humans associated with their presence in live animal markets in southern China [23]. However, the subsequent sampling of bats in China provided serological evidence of infection with SARS-like coronaviruses in bats of the genus *Rhinolophus* (i.e. horseshoe bats) in Hubei and Guangxi provinces, confirmed by PCR of faecal samples [24••]. Bats of the genus *Rhinolophus* are therefore the likely reservoir hosts for SARS-CoV-1, with onward transmission to civets and (perhaps raccoon dogs) that acted as 'intermediate' hosts to seed human infection [24••]. The presence of SARS-like coronaviruses with high sequence similarity to SARS-CoV-1 were later identified in Chinese horseshoe bats (*Rhinolophus sinicus*) [12••,25]. Notably, a study from multiple locations in China detected the conserved RNA-dependent RNA polymerase (RdRp) domain of coronaviruses in 6.5% of bat species from the genera *Rhinolophus*, *Pipistrellus*, *Scotophilus*, *Myotis*, *Tylonycteris* and *Miniopterus*, with phylogenetic analysis showing that three of the coronaviruses detected, all from the genus *Rhinolophus*, clustered with SARS-like coronaviruses [26].

In 2012 Middle Eastern Respiratory Syndrome coronavirus (MERS-CoV) appeared in Saudi Arabia, resulting in respiratory illness and relatively high levels of mortality [27]. Studies of Dromedary camels identified multiple MERS-CoV-like lineages (as well as the alphacoronavirus HCoV-229E) reflecting several decades of circulation in these animals with multiple transmission events to humans and widespread recombination [28]. Notably, MERS-like CoVs were also identified in multiple bat species (*Pipistrellus* cf. *hesperdus*, *Nycteris* cf. *gambiensis*, *Pipistrellus nathusii*, *Pipistrellus pipistrellus*, *Pipistrellus pygmaeus* and *Neoromica* cf. *zuluensis*) [29–31]. Hence, these bat viruses likely represent the reservoir wildlife hosts for the viruses that later emerged, via camels, as MERS-CoV. However, the bat coronaviruses most closely related to MERS-CoV also differed markedly in the spike protein and had reduced capacity to bind to the human dipeptidyl peptidase 4 cell receptor used by MERS-CoV [29,32•].

SARS-CoV-2 was first reported to cause severe pneumonia in humans in Wuhan, China in late 2019 [33•]. Metagenomic surveys and associated phylogenetic analyses have identified viruses closely related to SARS-CoV-2 in *Rhinolophus* bat species from several Asian countries (China, Cambodia, Thailand, Japan and Laos) [19••,20,21,34,35•,36]. For example, five SARS-CoV-2 related coronaviruses were detected in pooled faecal samples collected from bats (*Rhinolophus pusillus*, *Rhinolophus stheno* and *Rhinolophus malayanus*) in two studies of a single tropical botanical garden in Yunnan province, China [19••,20]. Close relatives of the alphacoronavirus porcine epidemic disease virus were identified at the same sampling site [19••]. Of most note, five SARS-CoV-2 related coronaviruses were recently identified in three *Rhinolophus* species in Laos (*R. malayanus*, *R. pusillus* and *Rhinolophus marshalli*), three of which grouped closely with human SARS-CoV-2 on phylogenetic trees and possessed a receptor binding domain with high sequence similarity to that of SARS-CoV-2 and the ability to bind to the human ACE2 receptor [21]. This provides strong evidence that viruses with the capacity to infect humans exist in wildlife species and hence represent a pandemic risk. Genomic recombination has also been commonplace among viruses of the SARS-CoV-2-like lineage (i.e. the sarbecoviruses) [19••,21,34,37], greatly complicating attempts to accurately reconstruct evolutionary history and suggesting that mixed infection is commonplace in bats and perhaps other mammalian species.

Although most of the focus has necessarily been on human disease, coronaviruses of bat ancestry have resulted in disease outbreaks in species other than

Figure 2



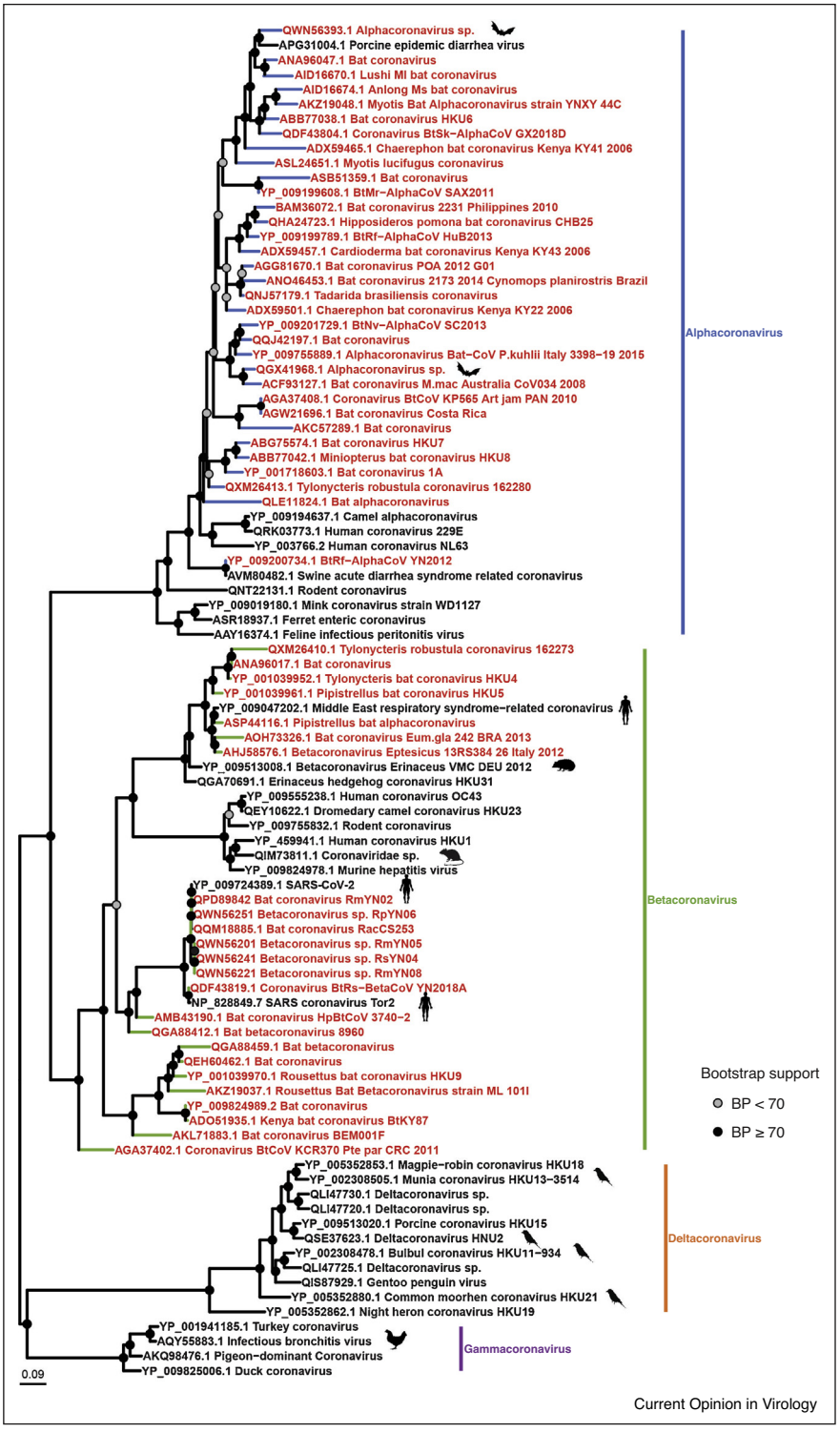
Number of publicly available virus gene sequences for each family of bats. The heat map shows **(a)** the number of RNA viruses and **(b)** the number of DNA viruses separated by virus family, and the corresponding bat host family as given on NCBI. Virus sequences with an unspecified bat host (i.e. host listed as Chiroptera) are represented as 'unclassified Chiroptera'.

humans. For example, a novel alphacoronavirus – Swine Acute Diarrhoea Syndrome coronavirus (SADS-CoV) – caused the death of upwards of 24 000 pigs in China in 2016 [38]. SADS-related coronaviruses were detected in rectal swabs from horseshoe bats in Guangdong province from 2013 to 2016 [38].

Paramyxoviridae

Interest in bat paramyxoviruses began with the discovery of Hendra, Nipah and Menangle viruses (see below). Since then, a large number of bat paramyxoviruses have been characterised, exhibiting considerable phylogenetic diversity (Figure 4). For example, 14 novel bat

Figure 3



Representative phylogenetic diversity of bat coronaviruses. An amino acid alignment of the RdRp of the *Coronaviridae* was used to infer a maximum likelihood phylogeny using IQ-TREE [81]. The phylogeny was estimated using viruses from the four coronavirus genera as marked by coloured lines to the right of the phylogeny. The tree was mid-point rooted and bootstrap values are represented by coloured circles. Bat viruses are shown in red font and fall into the *Alphacoronavirus* and *Betacoronavirus* genera. Animal silhouettes representing host species are displayed next to viruses that do not list a host species in the virus name.

Figure 4

Representative phylogenetic diversity of bat paramyxoviruses. An amino acid alignment of the L protein that contains the RdRp of the *Paramyxoviridae* was used to infer a maximum likelihood tree using IQ-TREE [81]. The tree was mid-point rooted and bootstrap values are represented by coloured circles. Bat viruses are shown in red font. Animal silhouettes representing animal host species are displayed next to viruses that do not provide a host species in the virus name. The avian paramyxovirus clade has been collapsed to enhance visualisation.

paramyxoviruses were discovered in nine species of bats in a single mNGS study from China [12••], while in 2020 a novel bat paramyxovirus – Achimota Pararubulavirus 3 – was identified in a urine sample from *Eidolon helvum* [39].

Hendra virus (genus *Henipavirus*, family *Paramyxoviridae*) was first reported to cause acute respiratory illness in horses and encephalitis in humans in Queensland, Australia in 1994, initially resulting in the death of 16 horses and two humans who had contact with sick horses [40–43]. After 2000 horses tested serologically negative to the virus it was proposed that it likely originated from a wildlife source [44,45], and the black flying fox (*Pteropus alecto*), grey headed flying fox (*Pteropus poliocephalus*), spectacled flying fox (*Pteropus conspicillatus*) and little red flying fox (*Pteropus scapulatus*) were later identified as carrying neutralising antibodies to the virus [45]. Hendra virus was later confirmed in the birthing fluid and foetal tissue from a grey headed flying fox and the foetal tissue from a black flying fox, as well as a high prevalence in bat urine [46,47]. Urine may therefore represent the likely mode of transmission to horses, with subsequent transmission through respiratory secretions to horses and humans [45–47].

Another member of the genus *Henipavirus* – Nipah virus – has similarly made the jump from bats to domestic animals. This virus was first reported in farmed pig populations in Malaysia in late 1998 before emerging in Singapore in early 1999 and again in India and Bangladesh in 2001, causing neurological and respiratory illness in pigs and encephalitis in humans [48,49]. During the 1998 Malaysia outbreak 238 humans contracted the virus, 105 of whom died [50]. Following the identification of Hendra virus in flying foxes (genus *Pteropus*), the large flying fox (*Pteropus vampyrus*), small flying fox (*Pteropus hypomelanus*), cave nectar bat (*Eonycteris spelaea*), lesser short-nosed fruit bat (*Cynopterus brachyotis*) and lesser Asiatic yellow bat (*Scotophilus kuhlii*) were identified as carrying neutralising antibodies to the virus [51]. The Indian flying fox (*Pteropus medius*, previously *Pteropus giganteus*) has similarly been linked to the Nipah virus outbreaks in India and Bangladesh [52,53].

The third zoonotic virus of the family *Paramyxoviridae* carried by bats is Menangle virus (genus *Pararubulavirus*). Menangle virus successfully established infection in a farmed pig population in 1997, presenting as reproductive complications [54]. Two humans working at separate piggeries recorded severe influenza-like symptoms during the 1997 piggery outbreak and were seropositive for Menangle virus [55]. Both workers confirmed they had been exposed to fluids from pigs housed at the outbreak farm [55]. Like Hendra, the grey headed flying fox, black flying fox and spectacled flying fox were later identified as carrying neutralising antibodies for Menangle virus and hence likely act as reservoir hosts [54,56], although no Menangle virus outbreaks have been recorded in Australia since 1997.

Rhabdoviridae

The *Rhabdoviridae* are a diverse set of negative-sense RNA viruses comprising multiple genera, one of which, the genus *Lyssavirus*, can cause rabies in mammals (the ecology and evolution of which has been extensively reviewed elsewhere [57]). Bats are the likely reservoir hosts for most lyssaviruses, although classic rabies lyssavirus is mainly transmitted to humans through bites or scratches from carnivores like dogs and racoons. Lyssaviruses currently include 17 characterised species that have been detected in bat species in a range of geographic locations, including Australian bat lyssavirus [58], Irkut lyssavirus (Russia) [59], Bokelon bat lyssavirus (Germany) [60], European bat lyssavirus 1 and 2 [61], Aravan and Khujand virus (Asia) [62] and Gannoruwa bat lyssavirus (Sri Lanka) [63]. Unfortunately, a lack of sampling makes it difficult to determine whether these viruses are present in other mammalian species.

For most bat species interactions with humans and other animals are limited to occasional occurrences such as through animal carers and in backyards and households. Accordingly, there is only sporadic lyssavirus transmission from bats to humans, although outcomes are often fatal. In contrast, the common vampire bat (*Desmodus rotundus*), hairy-legged vampire bat (*Diphylla ecaudata*) and white-winged vampire bat (*Diaemus youngi*) from South and Central America have a unique blood-feeding diet that provides an opportunistic route of the transmission for classic rabies lyssavirus into livestock [64].

Why are bats good reservoir hosts?

Bats undoubtedly harbour a large and diverse array of viruses, some of which have jumped species boundaries to emerge in new hosts and occasionally cause disease outbreaks. The question that naturally arises is why bats are seemingly such important reservoir hosts for zoonotic viruses, particularly as some studies indicate that the number of viruses carried by bats is significantly greater than other mammalian orders [65•]. The social dynamics of bat populations, including very large roosting numbers and species co-habitation, provide the perfect setting for viral transmission, while the capacity of bats to travel to different or new geographical regions provides a mechanism for viruses to become established in naïve bat populations. It is also likely that the characteristic flight-adapted physiology of bats in part provides an explanation for their high virus burden [66••,67], while the unique anti-inflammatory and proinflammatory responses in bats, as well as distinctive immunological traits such as the reduced number of interferon genes (such as in the black flying fox) and that the interferon genes are continually expressed in the absence of an initiated immune response, may in part explain why bats

are often asymptomatic carriers for a myriad of viruses [66••,67,68,69••]. More detailed studies of the innate and adaptive immune responses in a broader range of Yinpterochiroptera and Yangochiroptera should clearly be a research priority.

Despite the mounting evidence that bats harbour a particularly large and abundant virome, it is important to acknowledge that the increasing frequency with which bat viruses are described is also impacted by major ascertainment and confirmation biases. Indeed, following the discovery of the bat reservoir for SARS-CoV-1 there has been a marked increase in studies of bat viromes, with an emphasis on sampling from bat populations in China and other Asian countries [12••,19••,70,71•,72], and of particular genera such as *Rhinolophus*, although the population density of these animals varies markedly in space [19••]. Obviously, the more a particular group of animals is sampled then, on average, the more novel viruses will be characterised, and some studies have suggested that bats carry no more virus than expected given their species richness [73]. Perhaps more importantly, although virus species richness in bats is high, these viruses rarely establish successful human infection and most bat viruses classed as zoonotic are not directly transmitted from bats to humans. Rather, bat-to-human transmission routinely involves an intermediate animal host (such as pigs, camels and horses), and a number of the viral families that appear regularly in bat virome studies have not yet spilled over into human populations (Figure 2). Indeed, the zoonotic risk posed by bat viruses needs to be qualified by the observation that many of these viruses have been associated with bats for millennia with only a small number of spill-over events. For example, the common ancestor of the sarbecoviruses has been estimated to have existed ~21 000 years ago [74•], while the origin of the orthocoronaviruses has been dated to over 150 million years ago [75].

Challenges in bat viromics

The increasing use of mNGS has identified a multitude of novel and highly diverse RNA and DNA viruses in bats, greatly expanding our knowledge of the known virosphere and providing important information on the origins of specific viruses. The advantages of mNGS are manifold, including its unbiased and multiplex approach, easy application, high sensitivity and continually decreasing cost. However, the computational challenges of analysing the abundant sequence data produced by mNGS can be considerable, particularly in resource poor settings, and accurately identifying viruses that infect bats as opposed to components of their diet or microbiome can be challenging [76]. In particular, bats consume insects and are also susceptible to arthropod parasites, both of which may commonly carry viruses [77,78], and both insect viruses and bacteriophages are commonly detected in bat metagenomic data [5,8•,16,17]. Hence, to fully exploit the growing information obtained by mNGS studies of bat viromes new bioinformatics tools need to be developed that can rapidly and accurate identify those viruses most likely to infect bats. Similarly, determining which of the myriad of bat viruses are of likely human pandemic potential may not be possible though computational analyses alone. For example, although closely related to SARS-CoV-2, *Rhinolophus affinis* virus RaTG13 is unable to bind to the human ACE2 receptor [79].

Conclusions – an uncertain future for bats

Bats play a central role in maintaining a sustainable ecosystem, helping to pollinate, distribute seeds and control pests for thousands of plant species [80]. Since the identification of bats as reservoir hosts for many zoonotic viruses this group of animals has acquired an unjustified negative reputation, especially those species that roost in urban habitats, leading to an unsympathetic mindset among many communities. Climate change, urbanisation and industrial and agricultural advancements have greatly impacted bat populations globally [80], while encroachment onto bat habitats through urbanisation has increased the chance of viral spillover events into humans or companion and production animals. Currently, 106 bat species are listed as endangered or critically endangered on the IUCN (International Union for Conservation of Nature) red list, with 110 species threatened. Irrespective of any potential zoonotic risk, efforts to increase bat numbers should be prioritised. It is essential that we conserve this diverse group of animals, not only for the benefits to our ecosystem but also to enhance our understanding of viral biodiversity and evolution, as well as mammalian immunology.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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