Studies on the prevalence of viral pathogens in bat species inhabiting Wavul Galge cave, Sri Lanka

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Berlin, September 2022

Therese Muzeniek

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List of Abbreviations

ABLV	Australian bat lyssavirus
ACE2	Angiotensin-converting enzyme 2
AmpliSeq	Amplicon Sequencing
BLAST	Basic local alignment search tool
bp	Base pairs
CNS	Central nervous system
COI	Cytochrome c oxidase I
CoV	Coronavirus
EBLV	European bat lyssavirus
EBOV	Ebola virus
EID	Emerging infectious disease
GHPP	Global Health Protection Programme
HeV	Hendra virus
ICTV	International Committee on Taxonomy of Viruses
IDEA	Identification of Emerging Agents
IgG	Immunoglobulin M
IgM	Immunoglobulin M
LLOV	Lloviu virus
MARV	Marburg virus
MERS	Middle East respiratory syndrome
mNGS	Metagenomic NGS
MPXV	Monkeypox virus
MSA	Multiple sequence alignment
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NiV	Nipah virus
nt	Nucleotides
ONT	Oxford Nanopore Technologies
ORF	Open reading frame
PCR	Polymerase chain reaction
PMV	Paramyxovirus
RABV	Rabies virus
RBD	Receptor binding domain
RdRP	RNA-dependent RNA Polymerase
SARS	Severe acute respiratory syndrome

1. Zusammenfassung

Fledertiere der Ordnung *Chiroptera* sind weltweit verbreitet und gelten als natürliche Wirte einiger hochpathogener Viren. Die Zunahme durch virale Erreger ausgelöster Krankheitsausbrüche der letzten Jahrzehnte bewirkte auch eine gesteigerte Erforschung von Fledertieren und den mit ihnen assoziierten Viren.

Auch das zugrundeliegende Projekt dieser Arbeit befasst sich unter anderem mit Studien zur Prävalenz von Viren in Sri Lanka und deren möglichen Einfluss auf die Gesundheit der Bevölkerung. Der spezielle Fokus dieser Arbeit wurde dabei auf virologische Untersuchungen von Fledertieren der Spezies *Miniopterus fuliginosus*, *Rousettus leschenaultii, Rhinolophus rouxii*, *Hipposideros speoris* und *Hipposideros lankadiva* aus der Höhle Wavul Galge (Koslanda, Sri Lanka) gesetzt.

Die durchgeführte Arbeit hatte drei Hauptziele. Erstens wurden drei Feldstudien in der Höhle durchgeführt um die genannten Spezies zu fangen und zu beproben. Dabei wurde das Verfahren für zukünftige Einsätze optimiert, um virologische, mikrobiologische, zoologische und ökologische Forschungsaspekte zu berücksichtigen. Zweitens wurde eine Auswahl der gesammelten Proben mit verschiedenen molekularbiologischen Methoden untersucht und die Prävalenz von Viren in den Fledertieren nachgewiesen. Coronaviren und Paramyxoviren wurden mittels PCR in den Spezies *M. fuliginosus* und *R. leschenaultii* nachgewiesen. Proben der Spezies *M. fuliginosus* wurden weiterhin per NGS untersucht, aus den Daten wurde das Vollgenom eines α-Coronavirus assembliert, sowie die allgemeine Virom-Zusammensetzung und Präsenz weiterer neuer Virusstämme untersucht. Hierbei wurden weitere virale Sequenzen der Familien *Astroviridae*, *Coronaviridae*, *Iflaviridae*, *Paramyxoviridae*, *Picornaviridae*, und unklassifizierter *Riboviria* nachgewiesen.

Drittens wurden diese Sequenzdaten weitergehend analysiert und phylogenetische Berechnungen durchgeführt, anhand derer das humanpathogene Potenzial der identifizierten Viren vorläufig als gering eingestuft werden konnte.

Mit den Ergebnissen dieser Arbeit konnte der erste Nachweis verschiedener Viren aus sri-lankischen Fledertieren erbracht werden. Zukünftige Studien auf Basis dieser Arbeit können helfen die Kenntnis über solche Viren und deren Prävalenz in Sri Lanka zu erweitern.

2. Summary

Bats of the order *Chiroptera* are globally distributed mammals and known to be the reservoir of numerous human pathogenic viruses. The increasing emergence of infectious disease outbreaks in the past century has also enhanced the research on bats and their associated viruses. The underlying project of this thesis also aims to assess the prevalence of viruses in Sri Lanka and their possible impact on public health issues.

The focus of this thesis was the virological study of bats inhabiting Wavul Galge cave (Koslanda, Sri Lanka). The bats of the species *Miniopterus fuliginosus*, *Rousettus leschenaultii, Rhinolophus rouxii*, *Hipposideros speoris* and *Hipposideros lankadiva* live in one of the largest sympatric colonies that are known in Sri Lanka.

Three major aims were pursued in this work. First, three bat sampling sessions were conducted in Wavul Galge to collect different sample types from all bat species. In this context, the bat sampling was optimized for future studies to do structured and reasonable samplings by considering virological, microbial, zoological, ecological and further aspects.

Second, a variety of molecular virus detection methods was applied to estimate the prevalence of different viruses in the collected bat samples. Coronaviruses and Paramyxoviruses were identified in the bat species *M. fuliginosus* and *R. leschenaultii*. Furthermore, a full genome sequence of an α-Coronavirus derived from *M. fuliginosus* was assembled from NGS data. Also, a virome analysis of *M. fuliginosus* bats was obtained from mNGS data revealing the presence of further viruses. With this, viral sequences related to *Astroviridae, Coronaviridae, Iflaviridae, Paramyxoviridae, Picornaviridae* and unclassified *Riboviria* were detected.

Third, the obtained data were further used for in-depth sequence analysis, phylogenetic reconstruction and evaluation of the human pathogenic potential. Based on the available data, this human pathogenic potential was assessed to be rather low in all novel virus strains.

Summarized, the presented results of this thesis represent the first evidence of different viruses in cave-dwelling bat species from Sri Lanka. Further bat studies in this cave and other locations will increase the knowledge of virus prevalence in different bat species from Sri Lanka.

3. Introduction

3.1 Chiroptera – a species-rich and highly diverse order of mammals

Bats within the order *Chiroptera* are mammals that are present on every continent of the world except for the Antarctic and Arctic regions [1]. With unique abilities like flight and echolocation and a number of highly diverse features they are able to populate a lot of different trophic niches and environments, which prooves them to be a very successful order in terms of evolution [2]. Among all mammals, bats account for a proportion of approximately 20 % and are the second largest order after rodents [3].

Currently the order *Chiroptera* consists of 237 genera and approximately 1,447 living bat species according to current records of the Mammal Diversity Database (as of May 2022) [3, 4]. Originally, bats were classified based on their ability to echolocate, resulting in the suborders of echolocating *Microchiroptera* and non-echolocating *Megachiroptera* [5]. Based on extensive phylogenetic analysis of molecular data, *Chiroptera* are divided into *Yinpterochiroptera* and *Yangochiroptera*. In these two subgenera, both echolocating and non-echolocating bat species are represented [6].

This current taxonomy assigns the superfamily *Rhinolophoidea* and family *Pteropodidae* to the subgenus *Yinpterochiroptera*, while the subgenus *Yangochiroptera* consists of the three superfamilies *Emballonuroidea*, *Vespertilionoidea* and *Noctilionoidea* [7].

Based on molecular data and phylogenetic analysis of their flight and echolocation features, the order of bats probably developed in the Eocene period about 50 million years ago [5, 7]. Since then, bat genera have been evolving and diversifying with multiple different behavioral and morphological characteristics.

For example, their weight ranges between 2 g and 1.5 kg depending on the species which is associated with wing spans between a few centimeters or more than 1 meter in flying foxes [8]. A selection of the features that are exceptional for bats will be reviewed in the chapters 3.1.1 to 3.1.5.

3.1.1 Ability to fly

Bats are the only mammals that are capable of powered flight [1]. With this, they possess crucial advantages regarding ecological niches, forage and habitat and have the capability to migrate over long distances during seasonal changes [9]. As bats are predominantly nocturnal animals, echolocation or very advanced night vision enables them to coordinate their flights at night [8]. The ability to fly and associated migration behaviors may play an important role regarding disease transmission, but it may be also relevant in terms of co-existence with viruses, as will be discussed in the chapters 3.2.1 and 3.2.3 [1, 9].

3.1.2 Echolocation

Some, but not all bats perform echolocation for general orientation at night, identification of obstacles during flight and detection of prey [10]. This enables them to orientate, fly and hunt their prey even in complete darkness [8]. The echolocation signal is mostly produced in the larynx, while morphologically pronounced lips and noseleaves can enhance the outgoing signal [1].

Besides the mentioned function of orientation and hunting, echolocation may be used for communication between bats [1]. The main communication happens via social calls that are at a lower frequency than echolocation and also perceivable by humans [10]. These calls include a variety of signals such as distress and isolation calls, mating calls and copulation calls [1]. With echolocation, specific information such as sex, age and size of the bat may be communicated [10].

In general, the way of communication is very complex and individual for different bat species and families; the record and analysis of bat echolocation signals and social calls contributes to the ecological investigation of bats [10, 11].

3.1.3 Hibernation and daily torpor

Depending on species and latitude, bats have developed a wide range of adaptations to temperature conditions, ranging from hibernation over daily torpor to year-round homeothermy [12, 13].

Heterothermy describes the bats' ability to decrease their body temperature to almost ambient, which also decreases their metabolic rate to a minimum during that period [12]. Bats populating the north and south temperate zones use this mechanism for a longer hibernation period during winter time [1]. But bats in tropical and subtropical regions are also found to be heterothermic [12]. Bats in subtropical regions make use of shorter, multiday hibernation periods, while bats in tropical regions mainly use daily torpor in order to reduce their metabolic rate and to save energy [12]. Both mechanisms of hibernation and torpor have been suggested to contribute to the longevity of bats which is on average three times higher than in mammals of comparable size and metabolic rate [14].

3.1.4 Diet and contribution to ecosystems

With their high diversification bats populate a number of trophic niches, also in terms of their diet. Although most bats are insectivores, some species also feed on fruit, nectar, leaves, blood, fish and small vertebrates [8, 15]. These different dietary strategies result in extreme morphological diversity such as specific skull shapes and an adapted feeding apparatus [15]. While some bats and especially insectivorous representatives use the echolocation system for the detection of their prey, other non-echolocating bat families such as *Pteropodidae* have large eyes and good night vision in order to find their food consisting of fruit, flowers, nectar, pollen and leaves [1].

With their wide range of dietary habits bats contribute to their ecosystems in manifold ways, especially in tropical habitats. Insectivorous bats are important predators of insects and herbivores. By controlling the population sizes of agricultural pests, they indirectly support food production and increase crop yield [16, 17]. Calculations estimate that the bats' contribution to the food sector is worth several million USD per year [16, 18]. But non-insectivorous bats also play important roles in ecosystem preservation. Frugivorous bats are

important seed dispersers by transporting the seeds of plants and trees away from the parental trees. With this, they can contribute to forest regeneration in degraded landscapes [19]. For commercial plantations, fruit bats may reduce labor costs of seeding by taking over the seed dispersal [16].

Furthermore, frugivorous and nectivorous bats can contribute to the ecosystem by pollination of plants. Although birds and insects are more common in terms of pollination, bats are known to pollinate several hundred plant species [16, 19].

Another important role in ecosystems and agricultural use is bat guano which is rich in nutrients such as nitrogen and phosphorus. Guano from cave-dwelling bats serves as organic input for the flora and fauna within the cave [19]. In agricultural contexts, bat guano is used as natural fertilizer and improves plant growth when compared to chemical fertilizer [16].

3.1.5 Roosting and migration behavior

Bats live in colonies that can involve several million animals depending on the roosting site [1]. Roosts are resting places, protecting bats from predators and are important for social activities among the individuals [10]. Most bats worldwide live in caves, protecting the nocturnal animal from higher temperatures, predators and daylight during daytime [1]. In many cases caves are inhabited by multiple bat species forming large sympatric colonies, while the species usually occupy separate areas within the caves [20]. Furthermore, trees and foliage may be used as roosting sites. A prominent example are the large fruit bats of the family *Pteropodidae* that live in tropical areas; they can be observed hanging from tree branches during daytime and swarming out after sunset [1]. Colonies are usually limited in their number by the size of the trees, and fragmentation and relocation of smaller groups is a common process [1]. Especially in temperate zones synanthropic bat species are commonly found. As a substitute for natural roosts they populate buildings or roof spaces that are used as temporary shelters, maternity roosts or hibernation sites [21].

Bats may be facultative or obligate migrants and change their roosting sites depending on manifold external and intrinsic factors. Examples of external factors are the change of seasons and climatic conditions which may also be linked to changes in feeding opportunities. Bat species in temperate zones may migrate long distances between areas where they hibernate and

areas where they give birth to their offspring [22]. Some species show very specific migration patterns, living in smaller satellite colonies throughout the year and gathering in giant colonies for mating or maternity. Such movements and gatherings can have significant advantages for the colonies in terms of protection from predators and climate and rearing of offspring [8].
Generally speaking, the migration and movement patterns are as complex as the bat species are diverse, ranging from regional migration of tens of kilometers to long-distance migration of several thousand kilometers [1]. Prominent examples of long-distance migrating bats are flying foxes of the genus *Pteropus* that are capable of flying up to 2000 km in search of food and mating opportunities [23]. This results in a wide distribution of this bat genus ranging from Australia to Southeast-Asia and Eastern Africa [24]. Also, the bent-winged bat species of the genus *Miniopterus* is known for long-distance migration, resulting in a widespread distribution from Europe through Africa to Australia [1].
Independent of the intrinsic or extrinsic motivation, migration can result in very high dynamics within the bat populations and can be of interest regarding viral disease transmission.

3.1.6 Wavul Galge cave – roost for a sympatric colony in Sri Lanka

The thesis presented here includes virome studies on Sri Lankan bats inhabiting Wavul Galge cave. Therefore, the cave, the bat colony and the included bat species will be described in detail in the following. Wavul Galge (6°43'00"N 81°03'00"E) is an underground cave located in Koslanda, Sri Lanka (Figure 1) and harbors a sympatric colony of 100,000 individuals on average [25].

Figure 1: Location of Wavul Galge cave (marked in red) on the island of Sri Lanka (© OpenStreetMap in accordance with the Open Data Commons Open Database License).

Wavul Galge cave is a species-rich ecosystem and represents an important roosting site for thousands of bats. For bats of Sri Lanka exposed to tropical climate conditions, Wavul Galge cave provides optimal roosting conditions including stable temperature and humidity [8]. In addition, bats are protected from a number of predators; therefore the cave serves as ideal location for parturition and raising offspring.

Multiple bat species inhabit Wavul Galge cave, namely *Rhinolophus rouxii*, *Hipposideros speoris*, *Hipposideros lankadiva*, *Miniopterus fuliginosus* and *Rousettus leschenaultii*. Examining the behavior of the particular species and their sympatric interaction is of interest in many respects.

The bat species *R. leschenaultii* is the only fruit bat and the largest bat species roosting in Wavul Galge cave. In general, this species is of interest for zoological and ecological questions, as it is the only cave-dwelling and echolocating fruit bat [26]. With two reproductive cycles in April and September, the population size of *R. leschenaultii* undergoes a peak during that time [27]. The species *R. rouxii* also usually breeds in April to May and September to October. For this species, Wavul Galge is used as maternity roost and pregnant females migrate to the cave for parturition. This results in high fluctuations of the species' population size seasonally during the year [28].

Hipposideros bats generally show no migration behavior. This results in rather consistent population sizes during the year with peaks during the respective breeding times. For *H. speoris*, two breeding cycles in April to May and September to October are known. For *H. lankadiva*, a single breeding cycle takes place from March to May [29].

The migration behavior of *M. fuliginosus* is well studied in Sri Lanka. Pregnancy starts in mid-July and parturition is observed in late August, both having impact on the movement of mainly female *M. fuliginosus* bats. On the whole island of Sri Lanka, only Wavulpena cave was observed to serve as parturition and maternity cave for *M. fuliginosus*. Wavul Galge is a large cave approximately 50 km north of this cave and is used as pre-maternity roost for pregnant females throughout the migration procedure [30].

3.2 Bats as viral reservoirs

The combination of some of the characteristics mentioned in chapters 3.1.1 to 3.1.5, for example flying, migrating, distribution and longevity, makes bats a suitable reservoir host and susceptible to bacteria, parasites and a high number of viruses; this work will focus on the latter. The high diversity of viruses linked to the species-rich family of bats also presumes a long-time co-evolution between viruses and their hosts [20]. Consequently, bats as natural reservoirs must have evolved and developed strategies to escape severe viral infections.

3.2.1 Immunity of bats

The immune system of bats is basically similar to that of other mammals and may be divided into the innate and adaptive component. However, bats may have developed a number of mechanisms to host persisting viruses without developing diseases and severe infections. Due to their complexity, the response mechanisms have not been completely deciphered yet, but some features have already been identified or hypothesized to contribute to the unique immunity of bats towards viruses [31].

For example, the reduction of core body temperature to almost ambient in hibernating bats may be such a specific response mechanism. During the hibernation period, the metabolic rate as well as the replication of viruses is reduced and may support the long-term persistence of the virus at a low titer level [13].

Furthermore, the immune system of bats limits the release of pro-inflammatory signals. Compared to humans and other mammals, bats have lost a number of genes that are related to inflammatory gene families. Despite the apparent contradiction, the loss of these genes results in a more efficient and low intensity immune response with decreased impact on or harm to the bat hosts [32].

Other mammals including humans were discovered to change their gut microbiome composition and upregulate inflammation-associated genes with increasing age, which both contributes to the occurrence of age-related inflammations and diseases. These alterations are not observed in bats, which may also contribute to their longevity and insusceptibility to viral infections even at higher stages of age [33]. It is presumed that the general decrease of inflammatory processes in the innate immune system of bats in combination with gene expression for DNA repair, autophagy and tumor suppression substantially contributes to their longevity [34, 35].

Regarding the adaptive immune system, bats use immunoglobulins and cell-mediated immune response comparable to other mammals, although qualitative and quantitative differences are observed [36]. For instance, some bat species were found to transcribe IgM, IgE, IgA and IgG classes which are highly diverse and show a higher variety to that of other mammals [31, 37]. This larger repertoire makes bats less dependent on the long-lasting process of B cell affinity maturation which is needed in humans and other mammals to produce high-affinity specific

antibodies. As a result, antibody titers in bats are generally lower and contribute to persistent virus infections [31].
The described examples of innate and adaptive immune responses represent only some of the bats' mechanisms to escape a severe immune response after viral infection. The complexity and interaction of different pathways is not yet fully understood and therefore subject to current and future research on bats and viruses.

3.2.2 Emerging infectious diseases in bats

More than 70 % of all emerging infectious diseases (EIDs) are presumed to be zoonotic and can originate in livestock or wildlife, for example in bats and rodents [38, 39]. Although rodents have a generally higher species richness, bats host more zoonotic viruses per species [38]. Consequently, bats are being discussed to be special reservoirs for EIDs as a result of their unique features and immune system (compare chapters 3.1 and 3.2.1). With a proportion of 85 % most of the viral sequences that have been detected in bats are assigned to RNA viruses, and also the majority of EIDs are caused by RNA viruses [40].

The history of research on EIDs with bats as presumed natural reservoir can be dated back to the 1930s, but it has been receiving increasing attention in the past decades as a result of important EID events [40, 41]. Some of the most important examples of EIDs and their connection to bats as suspected reservoir will be described in the following chapters 3.2.2.1 to 3.2.2.4.

3.2.2.1 Lyssaviruses

The genus *Lyssavirus* belongs to the family *Rhabdoviridae* and comprises enveloped single-stranded negative-sense RNA viruses with a specific bullet-shaped morphology [42]. The disease Rabies, probably the most prominent and oldest EID reported, is caused by multiple members of the genus *Lyssavirus*, e.g. by Rabies virus (RABV), European bat lyssavirus -1 and -2 (EBLV-1, EBLV-2) and Australian bat lyssavirus (ABLV) [43]. The first RABV was isolated in 1931 and shortly after was connected to bats as the probable natural

host [44, 45]. Today, most rabies cases in humans worldwide are caused by transmission from cats or carnivores such as dogs and have a virtually 100 % fatality rate [46]. The predominant transmission route is via saliva which is transferred to the wound during biting [47].

Lyssaviruses are highly neurotropic and disseminate via the central nervous system (CNS). The incubation period is highly variable and lasts on average two months or sometimes up to years, depending on the proximity of the infected wound to the CNS. Afterwards, the virus spreads rapidly from the CNS to the salivary glands for further transmission and to various other tissues. The clinical period lasts on average between one and 10 days and is characterized by the development of encephalitis accompanied by “furious” or “paralytic” symptomatic behavior including insomnia, irritability and anxiety [48, 49].

Most rabies cases are caused by bites from infected dogs and the associated disease causes more than 50,000 deaths per year worldwide, predominantly in Africa and Asia [50]. However, the true number of infections and deaths related to rabies is very likely higher due to unrecorded cases.

The global burden of rabies since the early 1900s raised the attention and enhanced research, surveillance and vaccine development against RABV [48]. The availability of a RABV vaccination program for humans but also domestic and stray dogs supports the containment of rabies virus infections [51]. As a result, dog-mediated rabies cases were eliminated in Western Europe, North American countries and some South American countries, while it is still a high burden with more than 21,000 human deaths per year in Africa and more than 35,000 in Asia, mainly India with around 35 % of all deaths globally.

Still, RABV variants are found in several bat species in South America and are nowadays the main cause of human rabies cases in the western hemisphere [52]. In the eastern hemisphere, only non-rabies lyssaviruses such as ABLV, EBLV-1, EBLV-2 are present in bat species and associated with sporadic spillover events and human rabies cases [53, 54]. In Sri Lanka, lyssaviruses were detected in brain tissue of *Pteropus medius* bats [55].

The effect of lyssavirus infections in bats and circulation patterns in bat populations is not yet completely understood. In contrast to infections in other mammals including humans and dogs, bats are discussed to serve as lyssavirus carriers. In this function they may shed the virus for a longer time frame via the salivary glands and develop symptomatic infections of their CNS at

a later stage. With this mechanism, they may play an important role in lyssavirus evolution, distribution and ecology [48]. Therefore, further research is helpful for improved understanding, surveillance and prevention of bat-borne rabies cases and the development of further specific vaccines against bat lyssaviruses.

3.2.2.2 Filoviruses

Members of the family *Filoviridae* are enveloped negative-sense non-segmented RNA viruses and are further classified into eight genera [56]. The high virulence, the severe course of disease and high mortality in humans have raised the awareness of *Filoviridae* in the past decades [57]. Within this family, the three genera *Marburgvirus*, *Ebolavirus* and *Cuevavirus* are of special interest in the context of bat-borne and zoonotic viruses. Marburg virus (MARV) was the filovirus discovered first in 1967 and ever since caused sporadic outbreaks mainly in Africa. The largest MARV outbreak was recorded in Angola in 2004 to 2005, with a total of 252 infections and 227 fatalities [58]. The most prominent filovirus genus is *Ebolavirus* and comprises six different Ebolavirus species (EBOV), causing several outbreaks in African human communities since 1976. The largest EBOV epidemic occurred in 2014 to 2016 in West Africa with more than 11,000 deaths [47]. Both MARV and EBOV can cause severe hemorrhagic fever with high fatality rates in humans and non-human primates. The viruses are transmitted from animals to humans or directly between humans via blood or body fluids. In general, bats are presumed to be the natural hosts of these filoviruses [59].

For the EBOV outbreak in 2014 to 2016, the index case was traced back to close contact with insectivorous bats which are considered as natural hosts for EBOV [47, 60]. Furthermore, EBOV can be transmitted to humans via non-human primates which serve as intermediate host with a high mortality rate after being infected [61]. In the case of MARV outbreaks, epidemiological data show that 70 % of index cases may be linked to mining activities or visitation of caves that are inhabited by bats, assuming a viral shedding by the bats and possible transmission to humans via aerosols [62–65]. In subsequent studies, MARV, EBOV and related viruses have been detected in a variety of yinpterochiropteran and yangochiropteran bats by the detection of RNA and antibodies, respectively. While the geographical distribution of MARV and EBOV was long presumed to be restricted to central and western Africa, serological bat

studies reported the prevalence of further *Filoviridae* in Southeast Asia, including Bangladesh, China, the Philippines and India since the 2010s [57, 66–69].

Lloviu virus (LLOV) within the genus *Cuevavirus* was the first filovirus reported in Europe (Lloviu cave, Spain, 2011) and was also reported a second time in Hungary in 2016 [70, 71]. This filovirus is noteworthy as it was detected in five dead bats of the species *M. schreibersii*, but not in healthy individuals inhabiting Lloviu cave. Furthermore, nine dead bats of the co-roosting species *M. myotis* were also found but no filovirus sequences were detected in these individuals.

Consequently, this may be the first reported filovirus causing diseases in bats with probable host specificity for the species *M. schreibersii*, while MARV, EBOV and other filoviruses may persist in different bat species without causing any severe diseases [70].

Further research on filoviruses will be necessary to completely decipher the role of bats for filovirus distribution and spillover dynamics to primates and humans. Investigating the differences between LLOV and other filoviruses may help to better understand immunity mechanisms in bats.

3.2.2.3 Paramyxoviruses

The highly diverse family of *Paramyxoviridae* (PMV) consists of enveloped, negative-sense, non-segmented RNA viruses comprises 17 genera and is further divided into the subfamilies *Avulavirinae*, *Rubulavirinae*, *Orthoparamyxovirinae* and *Metaparamyxovirinae*. Viruses within the subfamily *Orthoparamyxovirinae* are assigned to seven genera and known to infect a broad range of hosts, such as reptiles, birds, fish and mammals [72]. Several human pathogenic viruses can be found within the genera of *Orthoparamyxovirinae*, such as Human respiroviruses, Measles virus, Hendra virus and Nipah virus, causing a spectrum of diseases with different severity. But a range of livestock, mainly swine and horses, are also susceptible to PMVs or were identified as intermediate hosts for human pathogenic PMVs [73].

Bats are considered and partly proven to be the natural reservoir for a number of PMVs that are themselves human pathogenic or related to such human pathogenic PMVs [74].

The most prominent PMVs associated with bats are Hendra (HeV) and Nipah virus (NiV) within the genus *Henipavirus*. Both viruses are highly pathogenic for humans and may cause

respiratory symptoms and encephalitis, whereas the distribution area and usual transmission routes were found to be different in HeV and NiV [75].

The first outbreak of HeV was reported in 1994 in Brisbane, Australia, when 20 horses and two humans were infected and developed respiratory symptoms, resulting in the death of one human and all horses [76, 77]. Shortly after, seropositivity for HeV was detected in different flying fox species and bats were linked as viral reservoir in further research [78, 79]. Since the first emergence, sporadic HeV outbreaks occurred in Australia, with a severe impact on infected horses and incidental transmission to humans having close contact. To date, seven human HeV infections and four fatalities were reported [73]. Recent studies in Australian *Pteropus* bats and horses discovered novel HeV variants and the presence of a novel genotype [80–82].

NiV was first reported in Malaysia in 1998 and in Singapore in 1999, when a novel respiratory disease infected pigs and pig farmers having close contact. The first outbreak caused 283 human infections and 109 fatalities and resulted in the slaughtering of more than 1 million pigs [83–86]. Further NiV outbreaks are reported in Bangladesh almost annually with a fatality rate of over 70 % [87]. In India outbreaks were reported in 2001 (case fatality of 74 %), 2007 (case fatality of 100 %) and 2018 (case fatality of 91 %) [88–90]. Here, food-borne transmission from fruit bats to humans via contaminated raw date palm sap was reported as well as direct air-borne transmission from humans to humans [91–93].

In the years after the discovery of HeV and NiV, numerous other novel PMVs were detected in bats, such as Menangle virus, Porcine rubulavirus, Tioman virus and Cedar virus, which are all suspected to be transmissible to intermediate hosts and also humans [94–97]. In addition, the number of yet unclassified PMVs from numerous bat host species has been continuously increasing for years. The high diversity of PMVs impedes attempts to decipher the genetic and phylogenetic relationships among the virus species [98]. Since the PMV family was established within the ICTV (International Committee on Taxonomy of Viruses) classification system, it was amended 10 times until 2019 by adding further subfamilies and genera [99, 100]. This proves that the attention to PMV research is constantly rising; yet many open questions are still to be resolved in this field.

3.2.2.4 Coronaviruses

Coronaviruses (CoV) belong to the subfamily *Orthocoronavirinae* within the family *Coronaviridae* which comprises a monophyletic cluster in the order *Nidovirales* [101]. CoVs are enveloped, positive-strand RNA viruses and can be divided into the genera of α-CoV, β-CoV, γ-CoV and δ-CoV. So far, only α-CoVs and β-CoVs have been detected in bats that are therefore considered as a natural reservoir, whereas γ-CoVs and δ-CoVs presumably originate from birds [102, 103]. To date, seven CoVs are known to infect humans, two in the α-CoV genus (HCoV-229E and HCoV-NL63) and five in the β-CoV genus (HCoV-OC43, HCoV-HKU1, SARS-CoV, MERS-CoV, SARS-CoV-2) [104].

Within the genus of α-CoVs, 15 subgenera and 26 virus species are currently assigned according to ICTV taxonomy (as of May 24, 2022) [101]. α-CoVs are known to infect a variety of mammals including humans, pigs, cats and bats. The first α-CoV species have been identified in bats of the genus *Miniopterus* in China; their co-infection of multiple diverse α-CoVs in one bat host suggests a long-term co-evolution of α-CoVs in *Miniopterus* bats [105]. Furthermore, genetically divergent α-CoVs have been detected in Chinese bats of the genera *Myotis*, *Scotophilus* and *Rhinolophus* [106]. Further CoV research in bats revealed the presence of α-CoVs in other Asian countries, and also different bat genera in Europe (e.g. *Pipistrellus, Nyctalus*), Africa (e.g. *Chaerephon, Cardioderma*) and North America (e.g. *Eptesicus, Perimyotis*) were found to host a high diversity of α-CoVs [106].

The human pathogenic α-CoV HCoV-229E was discovered in 1962 and is nowadays distributed worldwide causing the seasonal common cold [107, 108]. A closely related α-CoV was detected in a *Hipposideros caffer ruber* bat in Ghana in 2008. Further studies and phylogenetic data suggest that the viruses shared a most recent common ancestor approximately 200 years ago. Further data revealed that the virus was most probably transmitted from *Hipposideros* bats via camels as intermediate hosts to humans [109, 110].

HCoV-NL63 was detected in 2004 and is also connected to seasonally occurring mild respiratory diseases worldwide [111, 112]. Studies on *Perimyotis subflavus* bats in North America detected α-CoV sequences related to that of HCoV-NL63, and phylogenetic analysis predicted a most recent ancestor between 563 and 822 years ago, supporting the assumption of a bat host origin of that virus [113].

Within the genus β-CoV, five subgenera and 14 species are currently recognized, among which only the subgenera *Sarbecovirus*, *Merbecovirus*, *Nobecovirus* and *Hibecovirus* have also been detected in bats, while the subgenus *Embecovirus* is rather associated with rodent hosts [101, 102]. Two of the five human pathogenic β-CoVs, namely HCoV-OC43 and HCoV-HKU1, are causing the seasonal common cold with mild symptoms comparable to that of human pathogenic α-CoVs HCoV-229E and HCoV-NL63. Both HCoV-OC43 and HCoV-NL63 belong to the subgenus *Embecovirus* and have most likely originated in rodent hosts [114].

In contrast, the remaining three human pathogenic β-CoVs SARS-CoV, MERS-CoV and SARS-CoV-2 are capable of causing severe acute respiratory symptoms and spread easily from human to human. The severe acute respiratory syndrome (SARS) is caused by SARS-CoV (subgenus *Sarbecovirus*) and has induced the first global pandemic in the 21st century. Emerging in 2002 and 2003 in southern China, SARS-CoV has spread to 28 other countries, resulting in more than 8000 infections and almost 800 fatalities [115, 116]. Initial research in a live retail market in Guangdong, China, suggested an animal origin of the disease: mainly masked palm civets, but also raccoon dogs and Chinese ferret badgers were found to be seropositive for SARS-CoV [117]. Further investigation indicated that palm civets were not the natural reservoir host of SARS-CoV but were also susceptible to infections with a symptomatic course of disease [118, 119].

In search for the natural reservoir of SARS-CoV, *Rhinolophus* spp. in China were identified to carry SARS-like viruses and presumed to be the original host of SARS-CoV [120].

Subsequent research identified SARS-like viruses also in European and African bats [121–123]. Although related sequences were detected in *Hipposideros* spp. and *Chaerophon* spp., the highest prevalence of SARS-like viruses was found in *Rhinolophus* spp., supporting their significance as natural reservoir hosts.

MERS-CoV (subgenus *Merbecovirus*) was first reported in Saudi Arabia in 2012, causing 2,591 confirmed infections in humans and 894 fatalities worldwide (as of June 2022) [124]. Until today, the virus has been causing sporadic diseases, although limited to the Middle Eastern region. MERS-CoV seems to be less easily transmissible and causes fewer infections, although having a higher mortality compared to that during the preceding SARS-CoV pandemic in 2002 and 2003 [108]. Initially, bats were also anticipated as natural reservoir for MERS-CoV but subsequent findings identified camels as original hosts [125, 126].

The most recent and ongoing pandemic of SARS-CoV-2 exceeds by far the previous SARS-CoV and MERS-CoV incidences. As of August 2022 more than 574 million cases and over 6.3 million deaths have been reported globally, affecting countries all over the world [127]. The novel virus was initially named 2019-nCoV and later renamed SARS-CoV-2 by the responsible ICTV study group [128]. However, phylogenetic studies suggest that SARS-CoV-2 is not a descendent of SARS-CoV. The significant differences in the spike protein and receptor binding domain (RBD) binding to human angiotensin-converting enzyme 2 (ACE2) receptors contribute to the enhanced infectivity and transmissibility of SARS-CoV-2 replicating in the upper respiratory tract, leading to a pandemic of such an extent [129, 130].

Comparable to the emergence of SARS-CoV, the spillover event and first SARS-CoV-2 cases were linked to a retail market in Wuhan, China, in December 2019 [131, 132]. Consequently, *Rhinolophus* spp. were suspected to be the natural reservoir and further research supported the theory, showing an overall identity of 96 % between the genomes of SARS-CoV-2 and BatCoV RaTG13, a bat CoV identified in *Rhinolophus affinis* after the SARS-CoV outbreak in 2003 [132]. Additionally, SARS-CoV-2-like Pangolin-CoVs were identified in Malayan pangolins that were also traded on the Wuhan market.

A detailed sequence analysis of the Pangolin-CoV spike protein revealed that the RBD is more similar to SARS-CoV-2 than the RBD of BatCoV RaTG13 [133, 134]. Most probably, pangolins served as intermediate hosts, and the development of a spike protein capable of binding to human ACE2 receptors was the result of mutation and natural selection processes in the host [135].

But subsequent human-to-human transmissions also induced the natural selection processes and alterations of the SARS-CoV-2 genome. Single mutations, mainly in the spike protein, have been leading to the emergence of new SARS-CoV-2 variants which are impeding the surveillance, prevention, control and vaccine development for Covid-19 [136, 137]. Despite this, developments and advances including the emergence of the Omicron variant, infection of many people and advances in vaccination may raise hope that the SARS-CoV-2 pandemic will progress towards endemicity at some point [138, 139].

Nonetheless, SARS-CoV-2 is a paradigm of a zoonotic EID with pandemic dimensions. This emphasizes the importance of further research on viruses, hosts and spillover dynamics as well

as on the multidisciplinary connection between the research fields of virology, epidemiology, immunology and vaccine development.

3.2.3 Drivers of viral spillover and transmission in humans

Since the accumulation of disease outbreaks in humans during the last century, EIDs came into general focus and the number of reported cases has been rising significantly until today [39]. Still, zoonotic EIDs are most likely underreported, considering that spillover events tend to occur in rural areas where medical care and disease diagnostics are limited. Furthermore, zoonoses are probably not even recognized as such if the disease spectrum is either mild or if the disease causes fatality without human-to-human transmission [140].

As illustrated in the previous chapters 3.1 and 3.2, bats are suitable reservoirs of EIDs due to their unique morphological and behavioral features and are already known to harbor numerous zoonotic viruses.

The high species diversity of bats and their global distribution may be factors supporting the persistence of potentially zoonotic pathogens. Although bat species are generally distributed worldwide, a significantly higher species richness is observed in lower latitudes and decreases with latitude [141]. Concurrently, the number of bat-associated zoonotic viruses is increased in these areas as illustrated in Figure 2 [39].

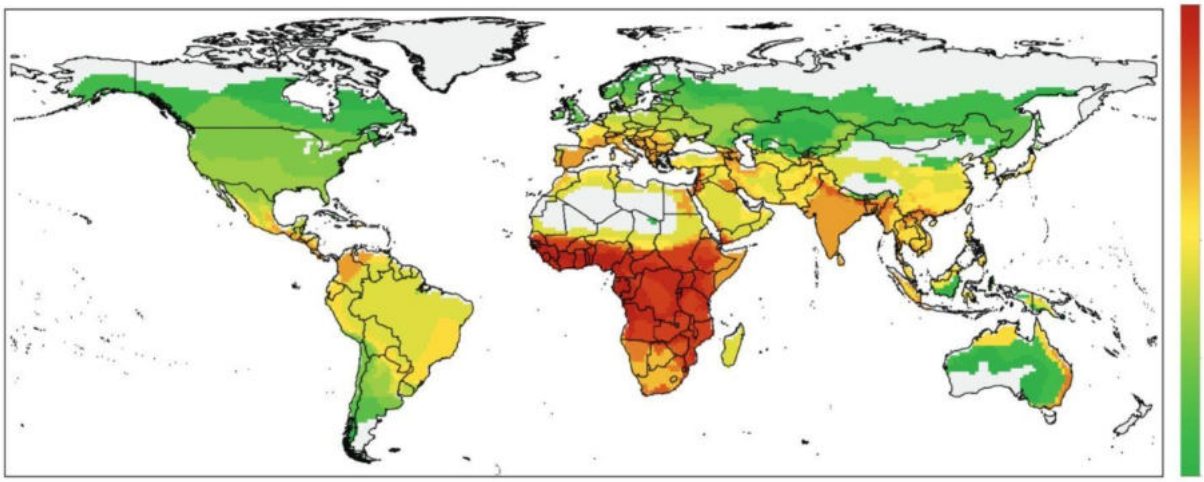

Figure 2: Schematic distribution of bat-associated zoonotic viruses worldwide. The colors represent a linear scale ranging from a low number (green) to a high number (red) of viruses in the respective area. Adapted from Brierley et al. [142].

The actual risk of zoonotic spillover events is mainly driven by factors and activities traced back to humans [143, 144].

One important factor for an increased spillover risk is close contact between humans and the infected animal. This can be a result of domestication, hunting, trading of animals and the general intrusion of humans into the natural habitat of wildlife including bats [140, 143].

These habitats are often destroyed by deforestation, infrastructural development and road construction for commercial or industrial purposes, such agricultural use, livestock farming or urbanization [39, 145]

Also, the human-made climate change and associated environmental changes bear a risk of viral spillover. Extreme weather conditions force bats in tropical countries to leave their natural habitat and settle in habitable regions which may be closer to the human residential areas. In these areas, the possible first-time contact to other animal species including livestock holds the risk of viral transmission between wildlife and livestock and emergence of new intermediate hosts of certain viruses [146].

Last but not least, the increasing globalization must be considered as driving factor of the introduction and fast dissemination of EIDs worldwide. The recent decades were characterized by increased travel and tourism and the development of complex transportation for international trade [147]. The dimensions of globalization were pointed out during the Covid-19 pandemic when international travelers spread the virus to countries all over the world and infection chains became impossible to retrace.

Outbreaks of severe EIDs in the past, such as SARS-CoV, Ebola virus and SARS-CoV-2, point out the importance of international collaboration, surveillance and epidemiology as well as enhanced research on pathogens, reservoir hosts and their interaction, while this research is especially important in countries where zoonoses are likely to emerge.

3.3 Investigation of bats as reservoir and research of associated viruses

The identification and characterization of reservoir species as well as researching the associated zoonotic viruses are important factors for monitoring, controlling and preventing EIDs [38].

For many decades, bats have been studied regarding their ecological behavior, echolocation, feeding, hibernation and other unique features. Their importance as natural hosts of zoonotic

viruses has raised awareness in the recent past, starting in the 1930s after identification of bats as reservoir of rabies virus [49].

This multidisciplinary field of research is investigated by experts in ecology, zoology, virology, molecular biology, immunology, epidemiology and numerous others.

Understanding the complex interrelations includes studying the behavior of bats, their distribution, the impact of climate change and human intrusion and their role as suitable reservoir. Also, deciphering whole genomes of further bat species will contribute to understanding the uniqueness of bats coping with virus infections and the mechanisms behind their immunity [148].

On the other hand, further research on viruses is of high importance to better understand host susceptibility, spillover dynamics and other factors regarding their human pathogenic potential. Currently (as of August 2022) more than 19,200 bat-associated viral sequences are deposited in the nucleotide sequence database of NCBI [149]. The more specific database DBatVir provides information on bat-associated viruses with a current record of 15,612 entries; the majority of around 88 % is represented by RNA viruses [150, 151]. However, the viral richness is probably many times higher and the current knowledge covers only a small proportion of the actual viral diversity in bats [38]. Anthony et al. studied the occurrence patterns in nine viral families and used an extrapolation approach to estimate a minimum of 320,000 yet undiscovered mammalian viruses [152]. Considering that there are far more families in the current viral taxonomy, the total number of undiscovered viruses is probably many times higher.

The focus of viral research in the last century was often biased as a result of EID events; this is still reflected in the proportions of virus sequence entries available. For example, about 30 % of the current sequence entries belong to *Rhabdoviridae* as a result of the global burden and high public health impact of rabies. Furthermore, 24 % of sequence entries belong to *Coronaviridae*, resulting from the increased interest in this viral family after the emergence of SARS-CoV, MERS-CoV and recently SARS-CoV-2. This research bias and the consequential high number of available data result from current public health issues but hold the general risk of underreporting other families that are not known or expected to cause diseases in humans [40, 153].

With the development of innovative virus detection methods, this bias in research may be reduced stepwise. Chapter 3.3.1 introduces detection approaches and associated methods that are generally used within the portfolio of virus research.

3.3.1 Virus detection and characterization methods

For the detection of viruses, especially in the context of viral diagnostics, numerous approaches and derived methods are available. These methods can be indirect and based on proving a past infection by detecting antibodies against the virus (e.g. IgM, IgG) with serological methods. Direct approaches include all methods that are actually detecting the virus or parts of it (nucleic acids, proteins) in a specimen. The available methods are highly variable regarding sensitivity and specificity, but also the costs, temporal expenditure, workload and required expertise are different for the approaches. Additionally the outcome of the respective method may range from the virus verification in a sample by indirect and direct methods to further typing of virus variants (e.g. serotypes, genotypes) up to the in-depth characterization of the detected viruses (e.g. viral genomics and transcriptomics).
A selection of common virus detection methods will be introduced in the following chapters 3.3.1.1 to 3.3.1.4.

3.3.1.1 Virus isolation using cell culture

The isolation of viruses in cell culture can be dated back to the 1960s; it replaced the more laborious and expensive method of infecting embryonated eggs and laboratory animals [154]. Since then, cell culture was considered as “gold standard” for virus detection for a long time until modern technology complemented and in part replaced this approach [155]. Cell culture can be used as a direct detection method as it proves the virus’ presence in a sample by infection of and proliferation in cultured cells. With this approach, cell culture is the only method to detect the presence of infectious particles in a sample. The successful virus isolation can be examined microscopically by observing alterations and degenerative changes on the cells. These cytopathic effects (CPE) can appear in different types and are very characteristic for different viruses or virus families, while some viruses do not cause any CPE when infecting

cells [156]. An important advantage of the method is that it can yield high concentrations of the infectious virus particles of interest; such isolates may be used for further analysis and characterization [157].

For successful virus isolation, the sample type and quality are essential. In contrast to other methods, infectious virus particles are necessary for the proliferation in cell culture. Therefore, the sampling effort, sample type, logistics and cold chain should be considered when planning viral studies that include cell culture approaches [158].

3.3.1.2 Detection of viral particles using electron microscopy

The negative staining transmission electron microscopy was long used in a diagnostic context for the rapid and direct detection of viruses in a specimen. The significant advantage of the method is the high genericity, which means that every virus species may be detected with this method and determined by means of characteristic morphological features [158].

An initial drawback of the method was its low sensitivity, which is continuously improved with the technical progress. The upstream ultracentrifugation of the sample or application of solid-phase immuno-electron microscopy are examples to increase the sensitivity of virus detection via electron microscopy [159]. Still, the method is mainly used for the diagnostics of skin lesion samples or for the gastroenteritis diagnostics of fecal samples, where high viral loads are expected [156]. As an open view method, important areas of applications are also in the context of viral research and bioterror diagnostics [158].

3.3.1.3 Protein-based detection of viruses

The protein-based detection of viruses may be divided into indirect and direct approaches as already described before (chapter 3.3.1). The underlying principle of direct methods is the binding of labeled antibodies to virus-associated proteins (antigens) [157]. For example, this can be realized with simple lateral flow tests as those now commonly used for the rapid detection of SARS-CoV-2 [160]. Although the method is very beneficial regarding easy sample preparation, fast and straightforward result interpretation and low costs, its main drawback lies

in the low sensitivity [157]. A more sensitive method for the direct detection of viral antigens is the ELISA (e.g. direct capture or sandwich method) which is commonly applied in laboratory-based viral diagnostics [161].

In contrast, indirect protein-based methods target antibodies that were generated by the host as response to viral infection [157]. Depending on the respective approach, a first characterization is already possible, i.e. differentiation of serotypes. The most common immunoassay is the ELISA technique, measuring the antibody response in the specimen to a given antigen. In the diagnostic and research context, these methods are widely used to identify recent or past exposure of a patient (or animal host) to a particular virus [161].

3.3.1.4 Detection of viral nucleic acids

The PCR method is the key element in terms of nucleic acid detection and has been used for decades for the detection of viral pathogens [157]. Compared to all previously described methods, the PCR is most sensitive and able to detect even low quantities of RNA and DNA in a sample. With its amplification approach, the nucleic acid sequence is multiplied up to an amount that can be visualized, e.g. on an agarose gel [158]. Compared to this conventional approach, modern real-time PCR techniques include fluorophore-labeled probes for an even more sensitive and faster real-time detection of nucleic acid amplification [162]. By using different fluorescent-labeled probes in a multiplex PCR assay for the simultaneous detection of different viruses, a broader screening with minimal effort is possible [163].

In both conventional and real-time PCRs, amplification of the target nucleic acid is realized by sequence-specific primers and requires a knowledge about the viral sequence. By targeting conserved regions of the viral genome and by using wobble bases in the primer sequences, slightly different viruses that for instance belong to the same group may be detected in one generic PCR assay [164]. With this approach the detection of novel viruses or virus strains within a known viral family is also possible, provided the sequence of the amplicon is determined subsequently.

For this purpose, traditional Sanger sequencing has been used for over 30 years to determine DNA sequences [165]. With this method, large sequencing projects were realized such as

sequencing the complete human genome. Typical read lengths for Sanger sequencing are 800 bp. If sequence information for whole genes of genomes is required, multiple PCRs are necessary with overlapping parts the sequences of which are combined in the subsequent bioinformatic data analysis. This laborious approach has impeded a high throughput of this method [153, 165].
In viral diagnostics, Sanger sequencing is also used for the detection of viral variants and different genotypes. For this purpose, often particular sequences such as highly variable gene sections are sufficient to determine different virus variants.

A more open-view technology is provided by Next Generation Sequencing (NGS) which can reveal sequence information of whole genomes, including RNA and DNA viruses, with a high throughput and comparably shorter time effort.

However, some challenges of NGS technology are the higher costs, especially in acquisition, and the required high expertise in molecular biology and bioinformatic data analysis [166]. With advances in technology the high costs and workload are stepwise reduced, while the increasing availability of user-friendly tools facilitates the data analysis [167].

By using suitable NGS sequencing platforms, high accuracy comparable to that of Sanger sequencing can be achieved [168]. Innovative 3rd generation sequencing technologies such as MinION (Oxford Nanopore Technologies, ONT) have become increasingly important for viral diagnostics questions as they provide comparably less expensive sequencing platforms accessible to a broader range of laboratories [166]. The initial drawbacks of low sequencing accuracy on these novel platforms are minimized with continual improvements of sequencing chemistry and can be furthermore compensated by using pre-amplification approaches. Pre-amplification and multiplex PCR techniques allow for the targeted sequencing of whole viral genomes or the screening for several viruses of interest in a panel on common NGS or 3rd generation sequencing platforms (including MinION) with a high sequencing accuracy [169, 170]. In the case of the Covid-19 pandemic, the MinION platform provides an affordable and easy-to-establish technology in laboratories for the sequencing of whole SARS-CoV-2 genomes which is used for surveillance and monitoring of new variants [170].

In addition to targeted detection approaches, different NGS or 3rd generation sequencing platforms including Roche Diagnostics (454), Life Technologies (Ion Torrent), Illumina (e.g. MiSeq, NextSeq) and MinION provide the possibility of untargeted so-called shotgun sequencing for the detection and de novo assembly of novel or unexpected virus sequences [166]. With this metagenomic NGS (mNGS) approach the general knowledge on existing viruses may be expanded. The obtained sequence data allow for further characterization of novel viruses, and the application of methods such as transcriptome analysis can be used to further study virus–host interactions [157].

In conclusion, the application of mNGS is a powerful tool in viral diagnostics and research. In theory it allows for an unbiased inspection of all viruses contained in a specimen (virome) and enables the discovery of novel emerging pathogens. Investigating the virome composition in a host may reveal its general health condition and also alterations due to diseases.

3.3.1.5 Comparison of virus detection methods

Nowadays, molecular and protein-based detection techniques dominate the field of virology, especially in viral diagnostics. But also conventional methods such as virus isolation in cell culture and electron microscopy are often used for further characterization, in particular of novel viruses, to obtain comprehensive knowledge about features such as size, morphology, propagation characteristics and more [157, 171]. Taking the respective advantages and drawbacks into account, the implementation of numerous virus detection methods can complement the knowledge about the novel virus. Table 1 gives a summary of the previously introduced virus detection methods regarding their sensitivity, specificity, workload and other characteristics that may influence the method selection.

Table 1: Tabular summary of previously introduced virus detection methods, comparing characteristics including sensitivity, specificity, required expertise, costs, time effort and the possibility to detect novel viruses. The evaluation ranges from Low over Medium to High and refers to the comparison of the introduced methods among each other.

	Virus isolation in cell culture
Detection principle	Virus propagation and observation of **CPE,** or PCR
Specificity	**Low** (e.g. in primary diagnostics) to **High** (e.g. transfection experiments)
Sensitivity	**High**
Detection of novel viruses?	**Yes**
Expertise, costs and time effort	**Medium** to **High** expertise, **Low** costs, **High** time effort
	Electron microscopy
Detection principle	Virus **visualization** and determination based on **morphology**
Specificity	**Low** (family level)
Sensitivity	**Low**
Detection of novel viruses?	**Yes**
Expertise, costs and time effort	**High** expertise, **High** costs, **High** time effort
	Protein-based virus detection (direct)
Detection principle	Binding and detection of **viral proteins** (**antigens)**
Specificity	**Medium** to **High** (depending on the target)
Sensitivity	**Low** to **Medium**
Detection of novel viruses?	**Partly** (detection of cross-reacting related viruses possible)
Expertise, costs and time effort	**Low** to **Medium** expertise, costs, time effort (depending on the method)
	Protein-based virus detection (indirect)
Detection principle	Detection of **antibodies** against the virus after infection
Specificity	**Medium** to **High**
Sensitivity	**Medium**
Detection of novel viruses?	**Partly** (detection of cross-reacting related viruses possible)
Expertise, costs and time effort	**Medium** expertise, costs and time effort (depending on the method)
	PCR
Detection principle	Detection of **viral nucleic acids**
Specificity	**Medium** to **High**
Sensitivity	**High**
Detection of novel viruses?	**Yes** (e.g. generic PCR assays)
Expertise, costs and time effort	**Low** to **Medium** expertise, costs and time effort (depending on the method)
	NGS
Detection principle	Detection of **viral nucleic acids**
Specificity	**Low**
Sensitivity	**Medium** to **High** (depending on sample type, background etc.)
Detection of novel viruses?	**Yes**
Expertise, costs and time effort	**High** expertise, costs and time effort (depending on the method)

3.3.1.6 Further characterization of detected viruses

Subsequent to virus detection, further characterization methods can be applied to assess the zoonotic and human pathogenic potential of a novel virus. In part, the previously introduced detection methods can already reveal particular characteristics of the novel virus. For example, virus isolation in cell culture may reveal the typical CPE patterns caused by the virus after infection of cells. In electron microscopy, characteristic morphological features may be discovered and serological studies may reveal cross-reactivity of antibodies to related antigens. A number of further characterizations can be performed with viral nucleic acid data derived from PCR and Sanger sequencing or NGS data sets. As described in chapter 3.3.1.4, these methods can reveal sequence information of partial genes or full genomes. With the help of mNGS the virome composition in a specimen can be analyzed. While Sanger sequences of PCR products may be analyzed with suitable software programs and the BLAST algorithm, mNGS data usually contain more complex data and require specific bioinformatic pipelines for thorough analysis. The workflows differ depending on the respective question (e.g. virome, microbiome, transcriptome analysis) but generally include an initial quality assessment of the data (e.g. adapter trimming), BLAST of data to a reference database (e.g. viral protein database via the diamond-alignment tool [172]) and visualization of results in a suitable program to select relevant sequences for further analysis (e.g. MEGAN [173]).

Comparative and phylogenetic analyses with other virus strains or species of the same family on nucleotide and amino acid sequence level can reveal information about the relationship and evolution of particular viruses.

Depending on the question, multiple sequence alignments (MSA) from partial sequences or ideally complete genes and open reading frames (ORF) can be calculated, and the in-depth analysis of such MSA can reveal structural and functional variations between related virus strains resulting from mutations in the nucleotide sequence and substitutions in the amino acid sequence of particular proteins [174]. In addition, the sequence-based allocation of a novel virus to a particular species is usually based on the comparative analysis of conserved viral genes, and the particular ICTV classification criteria are dependent on the respective virus family.

Calculating phylogenetic trees based on the MSA can illustrate hierarchical relationships of different virus strains assuming a common related ancestor [175]. A prerequisite for this is a

high quality alignment of a suitable sequence of the viral genome, for instance calculated by using the ClustalW algorithm [176, 177].

Phylogeny can be calculated by using different approaches such as Parsimony [178] or the more popular Neighbor-joining [179], Maximum-likelihood [180] and Bayesian methods [181, 182]. While Parsimony and Neighbor-joining approaches are basically simpler calculation methods, Maximum-likelihood and Bayesian methods use complex mathematical models for the calculation of phylogenetic trees [183]. In direct comparison, bootstrap probabilities as used for Maximum-likelihood calculations are less accurate compared to the Bayesian model which is a character state method that calculates posterior probabilities by adapting a set of relevant parameters in each generation of calculation [183–185]. These calculations can be visualized in phylogenetic trees in which the allocation to different branches represents the relationship of virus species or strains among each other as a function of the calculated probabilities. From this, important conclusions about the origin and evolution of a virus may be drawn which can also be important for epidemiology, disease surveillance or monitoring of outbreak situations.

4. Study design and methodology

4.1 IDEA project

This thesis was conducted in the context of the IDEA project (*Identification of Emerging Agents*) which is part of the Global Health Protection Programme (GHPP). The general goal of the IDEA project is to strengthen the diagnostic capacities for the detection of infectious diseases in the collaborating country Sri Lanka. Being an emerging economy, the country lags behind regarding current diagnostic standards and EIDs are likely to be underreported. Frequently, encephalitis cases are reported in human patients with unknown causative agents. Since Sri Lanka is a tropical country with a high biodiversity including bats and rodents, a zoonotic origin of these encephalitides appears to be likely. For instance, Nipah virus infections transmitted from pteropid bats to humans are frequently reported in the neighboring country India and other Asian countries in this region [87, 186, 187]. Although zoology is a highly recognized research field in Sri Lanka, only few investigations on wildlife animals were performed regarding their role as reservoir for zoonotic viruses.

At the same time, Sri Lanka has been experiencing a thriving tourism in the past decades, resulting in intrusion into yet unspoiled nature and increasing human proximity to wildlife. Therefore, the IDEA project focuses also on studying possible reservoir hosts such as rodents, bats and flying foxes. Using different approaches, the prevalence and persistence of viruses is investigated [188].

4.2 Aims of this thesis within the project

Regarding virology, only few investigations have been carried out on Sri Lankan bats so far [55, 189]. Therefore, the main aim of this thesis within the project is to thoroughly investigate the prevalence of viruses in different bat species from Sri Lanka for the first time. In contrast to previous studies, these investigations aim to include not only PCR-based screening methods but also NGS data for an unbiased virus discovery. With this, an initial attempt towards closing the gap of data knowledge in this country is envisaged.

This thesis focuses on virological investigations in Wavul Galge cave which is one of the largest in Sri Lanka and accommodates a sympatry of bats belonging to the species *M. fuliginosus, R. rouxii, H. speoris, H. lankadiva* and *R. leschenaultii* (compare chapter 3.1.6). In terms of zoology, the colony is an interesting object of investigation regarding the interaction of the different species among each other (e.g. roost allocation in the cave, forage competition), the migration behavior of some of the species (*M. fuliginosus, R. rouxii*) and the general population dynamics throughout the year. But also regarding virological questions, the sympatric bat colony is a relevant research object in many respects. The general aims of the conducted investigation are illustrated in Figure 3.

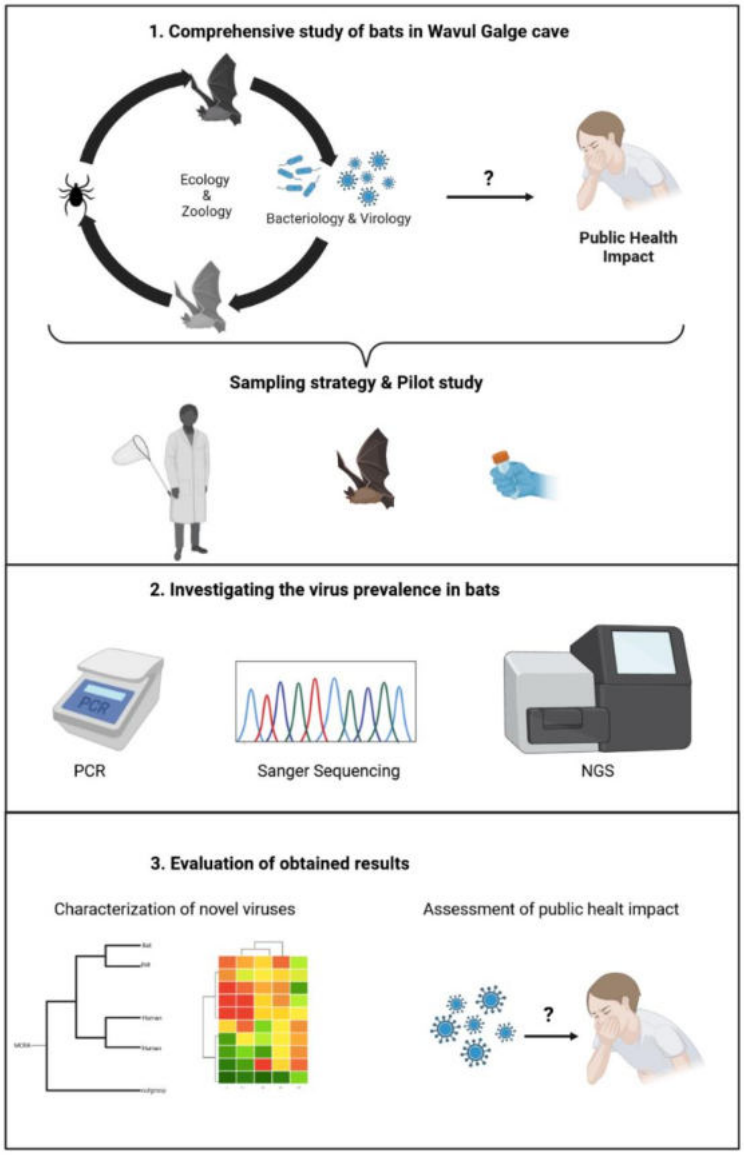

Figure 3: Schematic overview of the aims of the thesis. Created with BioRender.com

1. Comprehensive study of bats in Wavul Galge cave

The investigation of different bat species inhabiting Wavul Galge cave aimed to include questions comprising the research fields of ecology, zoology, virology and epidemiology. In order to fulfill this multidisciplinary approach, a joint strategy for capturing and sampling of bats had to be established (Figure 3; section 1).
This strategy was implemented and optimized in a pilot study consisting of three separate bat samplings at different times of the year (see also Figure 4).

2. Investigating the virus prevalence in bats

Samples obtained from the samplings were processed and analyzed to determine the prevalence of viruses in the bats. Different virus detection approaches were used, while this thesis focusses on the detection of viral nucleic acids. With generic PCRs and metagenomic NGS methods, the aim was to detect particular novel virus strains and to discover the general virome composition with an unbiased view (Figure 3; section 2).

3. Evaluation of the obtained results

The obtained results were further analyzed and the detected viruses characterized with in-depth sequence analysis and phylogenetic methods. The aim was to evaluate the zoonotic potential of newly discovered viruses. In conclusion, their impact on public health was assessed (Figure 3; section 3).

4.3 General methodology for the work of this thesis

To reach the presented aims of this thesis (see chapter 4.2), a total of three bat sampling sessions were conducted in Wavul Galge cave. However, not only the aims of the thesis but different other analyses were envisaged to be conducted with the collected bat samples. To give an overview, a summarized workflow of the bat sampling sessions and subsequent sample analyses (conducted and planned) is illustrated in Figure 4.

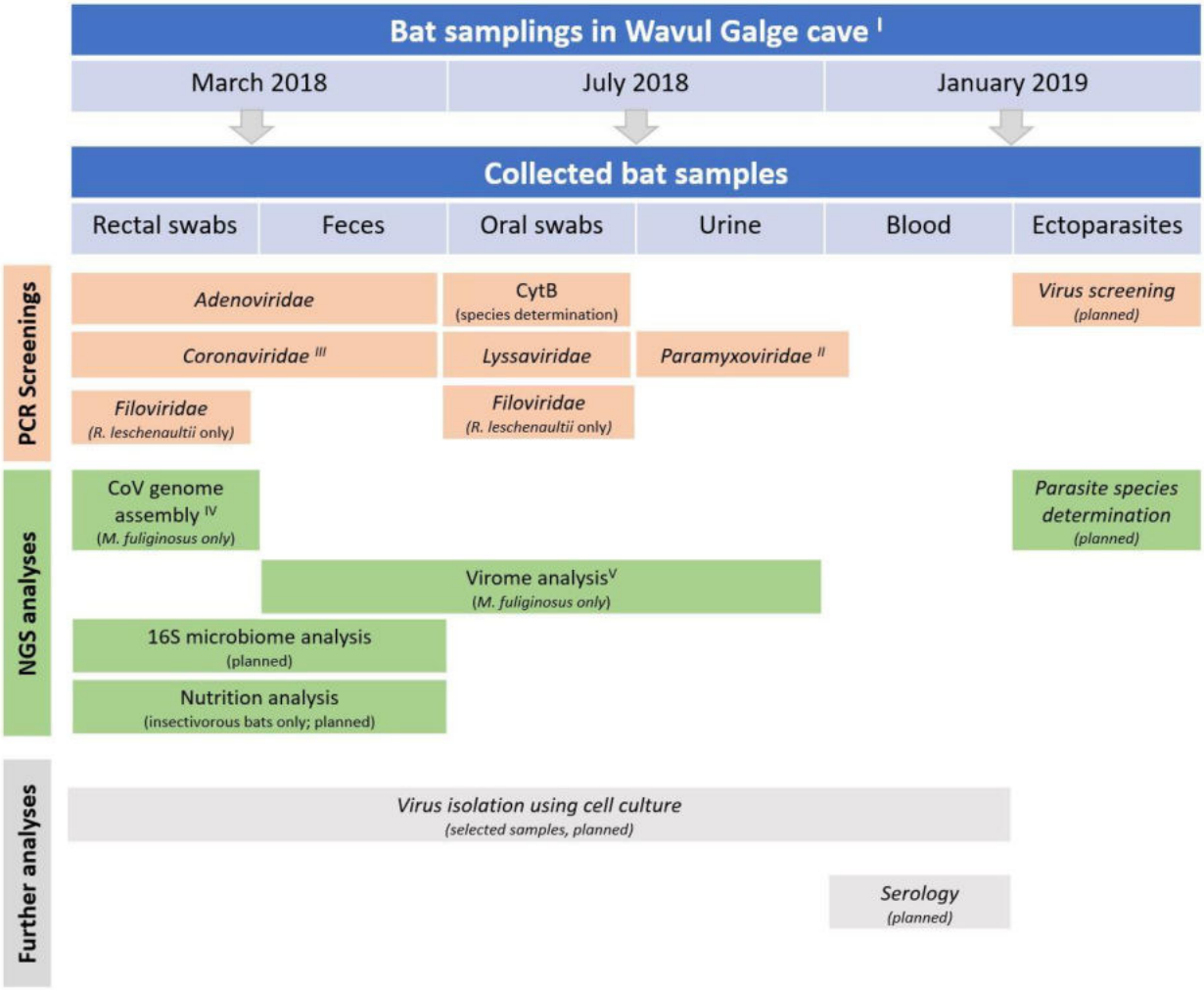

Figure 4: Overview of the three bat sampling sessions in Wavul Galge cave, collected samples and conducted or planned analyses with focus on the PCR screenings and NGS analyses. The workflow also indicates the works that contributed to the publications included in this thesis (indicated with roman numerals I–V for the respective publications, see chapter 5).

In order to cover different seasons of the year and potential seasonal patterns of viral shedding, samplings were planned for March 2018, July 2018 and January 2019. Bat sampling aimed to include oral swabs, rectal swabs, fecal pellets, urine swabs, blood and ectoparasites depending on availability and feasibility. The conducted samplings and gained experiences were to be used to develop a comprehensive sampling strategy and to facilitate the planning of future bat samplings (Publication I, see chapter 5).

Selected samples were analyzed by using different molecular biological methods. This included PCR screenings for particular viruses depending on the suspected viral shedding route (e.g. oral swabs for *Lyssaviridae*, rectal swabs and feces for *Coronaviridae* and *Adenoviridae* and urine for *Paramyxoviridae*) and in part only for the suspected bat hosts (e.g. oral swabs and rectal swabs from only *R. leschenaultii* for *Filoviridae*). Selected PCR screening results (CoV, PMV) are part of this thesis (Publication II–III, see chapter 5)

Furthermore, a number of NGS-based analyses are planned with the collected samples. This thesis focusses on the NGS analysis of samples from *M. fuliginosus*. This bat species shows migratory behavior and is distributed worldwide; it is also known to host a number of different viruses that are assumed to have human pathogenic potential (see chapter 3.2). From the obtained NGS data for *M. fuliginosus* rectal swab samples, a full genome of a novel CoV strain was assembled and characterized (Publication IV, see chapter 5). Virome analysis was also conducted with feces, oral swabs and urine samples collected from *M. fuliginosus* in July 2018; this time of the year represents a population peak of this species in Wavul Galge cave (Publication V, see chapter 5).

A number of further analyses are envisaged for the collected samples to resolve scientific questions of the research fields of zoology and microbiology (examples are given in Figure 4).

5. Summary of results

5.1 Overview of published results and particular contributions

The contributing publications are not listed chronologically, but in the order they are discussed in this thesis. For each publication, the respective contribution is stated regarding the concept and planning of conducted methods, execution of experiments, analysis of results and writing of the manuscript. A summary of the contribution is given in Table 2.

Publication I

One Health Approach for the sampling of different bat species living in a sympatric colony

Thejanee Perera*, Sahan Siriwardana*, Therese Muzeniek*, Beate Becker-Ziaja, Dilara Bas, Fatimanur Bayram, Mizgin Öruc, Inoka Perera, Jagathpriya Weerasena, Shiroma Handunnetti, Franziska Schwarz, Gayani Premawansa, Sunil Premawansa, Wipula Yapa, Andreas Nitsche and Claudia Kohl
* equal contribution

This publication was published online on 23 September 2022 at bioRxiv
DOI: https://doi.org/10.1101/2022.09.22.508887

Personal contributions:
Planning, preparation, conduction and optimization of bat sampling sessions in Wavul Galge cave, Sri Lanka, as part of the research team. Development of a sampling strategy for future samplings. Major contribution to the preparation and writing of the manuscript.

Publication II

Paramyxovirus Diversity within One Population of* Miniopterus fuliginosus *Bats in Sri Lanka

Therese Muzeniek*, Thejanee Perera*, Sahan Siriwardana, Fatimanur Kaplan, Dilara Bas, Mizgin Öruc, Beate Becker-Ziaja, Inoka Perera, Jagathpriya Weerasena, Shiroma Handunnetti, Franziska Schwarz, Gayani Premawansa, Sunil Premawansa, Wipula Yapa, Andreas Nitsche and Claudia Kohl
* equal contribution

This publication was published online on 2 April 2022 in:
Pathogens, Volume 11 (434)
DOI: https://doi.org/10.3390/pathogens11040434

Personal contributions:
Partial contribution to the planning of the PMV PCR screening of selected bat samples. Partial execution of experiments (PCR, sequencing). Major contribution to the analysis and visualization of obtained results (sequence analysis, phylogenetic reconstructions). Major contribution to the preparation and writing of the manuscript.

Publication III

Detection of Alpha- and Betacoronaviruses in* Miniopterus fuliginosus *and* Rousettus leschenaultii*, two species of Sri Lankan Bats

Therese Muzeniek, Thejanee Perera, Sahan Siriwardana, Dilara Bas, Fatimanur Kaplan, Mizgin Öruc, Beate Becker-Ziaja, Franziska Schwarz, Gayani Premawansa, Sunil Premawansa, Inoka Perera, Wipula Yapa, Andreas Nitsche and Claudia Kohl

This publication was published online on 15 June 2021 in:
Vaccines, Volume 9 (650)
DOI: https://doi.org/10.3390/vaccines9060650

Personal contributions:
Planning of the CoV PCR screening of selected bat samples. Major execution of experiments (PCR, sequencing). Major contribution to the analysis and visualization of obtained results (sequence analysis, phylogenetic reconstructions). Major contribution to the preparation and writing of the manuscript.

Publication IV

Full Genome of batCoV/MinFul/2018/SriLanka, a Novel Alpha-Coronavirus Detected in* Miniopterus fuliginosus*, Sri Lanka

Therese Muzeniek, Thejanee Perera, Sahan Siriwardana, Dilara Bas, Fatimanur Kaplan, Mizgin Öruc, Beate Becker-Ziaja, Inoka Perera, Jagathpriya Weerasena, Shiroma Handunnetti, Franziska Schwarz, Gayani Premawansa, Sunil Premawansa, Wipula Yapa, Andreas Nitsche and Claudia Kohl

This publication was published online on 7 February 2022 in:
Viruses, Volume 14 (337)
DOI: https://doi.org/10.3390/v14020337

Personal contributions:

Major contribution to the planning of experiments and applied analysis methods. Major execution of experiments (sample preparation for NGS, PCRs). Major contribution to the analysis and visualization of obtained results (NGS data analysis, Sanger sequence analysis, primer design, gene annotation, phylogenetic reconstructions). Major contribution to the preparation and writing of the manuscript.

Publication V

Comparative virome analysis of individual shedding routes of* Miniopterus fuliginosus *bats inhabiting the Wavul Galge Cave, Sri Lanka

Therese Muzeniek, Thejanee Perera, Sahan Siriwardana, Dilara Bas, Fatimanur Bayram, Mizgin Öruc, Beate Becker-Ziaja, Inoka Perera, Jagathpriya Weerasena, Shiroma Handunnetti, Franziska Schwarz, Gayani Premawansa, Sunil Premawansa, Wipula Yapa, Andreas Nitsche and Claudia Kohl

This publication was published online on 22 September 2022 at bioRxiv
DOI: https://doi.org/10.1101/2022.09.21.508883

Personal contributions:
Major contribution to the planning of experiments and applied analysis methods. Major execution of experiments (sample preparation for mNGS). Major contribution to the analysis and visualization of obtained results (NGS data analysis, visualization of virome data, primer design, phylogenetic reconstructions). Major contribution to the preparation and writing of the manuscript.

Table 2: Summary of the particular contributions to the publication that are part of this thesis

Publication	Authorship	Concept and planning of methods	Execution of experiments and analysis	Writing of the manuscript
I	Shared First	30 %	30 %	90 %
II	Shared First	50 %	60 %	90 %
III	First	60 %	70 %	90 %
IV	First	80 %	90 %	90 %
V	First	80 %	90 %	90 %

The abstracts of the publications are listed in the following chapters 5.2 to 5.6. The complete publications can be found in the Annex (12).

5.2 Abstract Publication I

One Health Approach for the sampling of different bat species living in a sympatric colony

Published as preprint at bioRxiv (https://doi.org/10.1101/2022.09.22.508887)

Bats are important contributors to the global ecosystems; at the same time, they are known to be a natural reservoir host for a number of human pathogenic viruses. These and many other unique features make them an interdisciplinary research object in the context of One Health, comprising zoology, ecology, virology, microbiology, molecular biology, immunology and public health issues. Performing field studies for bat research often aims to cover several of these topics and requires the combination of specific expertise in different fields. We carried out three individual field studies in Wavul Galge cave (Koslanda, Sri Lanka), where several bat species roost sympatrically. The main goals were to study the bat colony for ecological aspects and to sample bats for virological and molecular biological analyses. In the course of the field studies, we optimized the sampling procedure regarding safety aspects, a preferably low impact on the captured bats and an improved output of high-quality samples for further analysis. Different sampling methods and procedures were compared in order to establish a suitable strategy for frequent sampling and monitoring of these bats. In the present case study, we report on this process of optimizing our field work and provide suggestions for bat sampling methods that cause comparably less stress for the captured animals. We also report on constraints and obstacles encountered during the practical implementation and possible measures to overcome these.

With these practical experiences, we hope to give support to other interdisciplinary research teams preparing for bat field work. Furthermore, we emphasize the need for the respectful treatment of the animals and minimized disturbance of their natural habitat when carrying out sustainable bat research.

5.3 Abstract Publication II

Paramyxovirus Diversity within One Population of *Miniopterus fuliginosus* Bats in Sri Lanka

Published in Pathogens, Volume 11 (434) (https://doi.org/10.3390/pathogens11040434)

Bats are known as typical reservoirs for a number of viruses, including viruses of the family Paramyxoviridae. Representatives of the subfamily Orthoparamyxovirinae are distributed worldwide and can cause mild to fatal diseases when infecting humans. The research on Paramyxoviruses (PMVs) from different bat hosts all over the world aims to understand the diversity, evolution and distribution of these viruses and to assess their zoonotic potential. A high number of yet unclassified PMVs from bats are recorded. In our study, we investigated bat species from the families Rhinolophidae, Hipposiderae, Pteropodidae and Miniopteridae that are roosting sympatrically in the Wavul Galge cave (Koslanda, Sri Lanka). The sampling at three time points (March and July 2018; January 2019) and screening for PMVs with a generic PCR shows the presence of different novel PMVs in 10 urine samples collected from *Miniopterus fuliginosus*. Sequence analysis revealed a high similarity of the novel strains among each other and to other unclassified PMVs collected from *Miniopterus* bats. In this study, we present the first detection of PMVs in Sri Lanka and the presence of PMVs in the bat species *M. fuliginosus* for the first time.

5.4 Abstract Publication III

Detection of Alpha- and Betacoronaviruses in *Miniopterus fuliginosus* and *Rousettus leschenaultii*, two species of Sri Lankan Bats

Published in Vaccines, Volume 9 (650) (https://doi.org/10.3390/vaccines9060650)

Bats are known to be potential reservoirs of numerous human-pathogenic viruses. They have been identified as natural hosts for coronaviruses, causing Severe Acute Respiratory Syndrome (SARS) in humans. Since the emergence of SARS-CoV-2 in 2019 interest in the prevalence of coronaviruses in bats was newly raised. In this study we investigated different bat species living in a sympatric colony in the Wavul Galge cave (Koslanda, Sri Lanka). In three field sessions (in 2018 and 2019), 395 bats were captured (*Miniopterus*, *Rousettus*, *Hipposideros* and *Rhinolophus* spp.) and either rectal swabs or fecal samples were collected. From these overall 396 rectal swab and fecal samples, the screening for coronaviruses with nested PCR resulted in 33 positive samples, 31 of which originated from *Miniopterus fuliginosus* and two from *Rousettus leschenaultii*. Sanger sequencing and phylogenetic analysis of the obtained 384-nt fragment of the RNA-dependent RNA polymerase revealed that the examined *M. fuliginosus* bats excrete alphacoronaviruses and the examined *R. leschenaultii* bats excrete betacoronaviruses. Despite the sympatric roosting habitat, the coronaviruses showed host specificity and seemed to be limited to one species. Our results represent an important basis to better understand the prevalence of coronaviruses in Sri Lankan bats and may provide a basis for pursuing studies on particular bat species of interest.

5.5 Abstract Publication IV

Full Genome of batCoV/MinFul/2018/SriLanka, a Novel Alpha-Coronavirus Detected in *Miniopterus fuliginosus*, Sri Lanka

Published in Viruses, Volume 14 (337) (https://doi.org/10.3390/v14020337)

Coronaviruses (CoV) are divided into the genera α-CoVs, β-CoVs, γ-CoVs and δ-CoVs. Of these, α-CoVs and β-CoVs are solely capable of causing infections in humans, resulting in mild to severe respiratory symptoms. Bats have been identified as natural reservoir hosts for CoVs belonging to these two genera. Consequently, research on bat populations, CoV prevalence in bats and genetic characterization of bat CoVs is of special interest to investigate the potential transmission risks. We present the genome sequence of a novel α-CoV strain detected in rectal swab samples of *Miniopterus fuliginosus* bats from a colony in the Wavul Galge cave (Koslanda, Sri Lanka). The novel strain is highly similar to Miniopterus bat coronavirus 1, an α-CoV located in the subgenus of Minunacoviruses. Phylogenetic reconstruction revealed a high identity of the novel strain to other α-CoVs derived from Miniopterus bats, while human-pathogenic α-CoV strains like HCoV-229E and HCoV-NL63 were more distantly related. Comparison with selected bat-related and human- pathogenic strains of the β-CoV genus showed low identities of ~40%. Analyses of the different genes on nucleotide and amino acid level revealed that the non-structural ORF1a/1b are more conserved among α-CoVs and β-CoVs, while there are higher variations in the structural proteins known to be important for host specificity. The novel strain was named batCoV/Min-Ful/2018/SriLanka and had a prevalence of 50% (66/130) in rectal swab samples and 58% (61/104) in feces samples that were collected from Miniopterus bats in Wavul Galge cave. Based on the differences between strain batCoV/MinFul/2018/SriLanka and human-pathogenic α-CoVs and β-CoVs, we conclude that there is a rather low transmission risk to humans. Further studies in the Wavul Galge cave and at other locations in Sri Lanka will give more detailed information about the prevalence of this virus.

5.6 Abstract Publication V

Comparative virome analysis of individual shedding routes of *Miniopterus fuliginosus* bats inhabiting the Wavul Galge Cave, Sri Lanka

Published as preprint at bioRxiv (https://doi.org/10.1101/2022.09.21.508883)

Bats are described as the natural reservoir host for a wide range of viruses. Although an increasing number of bat-associated, potentially human pathogenic viruses were discovered in the past, the full picture of the bat viromes is not explored yet. In this study, the virome composition from *Miniopterus fuliginosus* bats inhabiting the Wavul Galge cave, Sri Lanka, was analyzed. To assess different possible shedding routes, oral swabs, feces and urine were collected and analyzed individually by using metagenomic NGS. The data obtained was further evaluated by using phylogenetic reconstructions.

Two different alphacoronavirus strains were detected in feces and urine samples. Furthermore, a paramyxovirus was detected in urine samples. Sequences related to *Picornaviridae, Iflaviridae,* unclassified *Riboviria* and *Astroviridae* were identified in feces samples, and further sequences related to *Astroviridae* in urine samples. No further viruses were detected in oral swab samples.

The comparative virome analysis in this study revealed a diversity in the virome composition between the collected sample types which also represent different potential shedding routes for the detected viruses. At the same time, several viruses were detected for the first time in bats in Sri Lanka.

The detection of two different coronaviruses in the samples indicates the potential general persistence of this virus species in *M. fuliginosus* bats. Based on phylogenetics, the identified viruses are closer related to bat-associated viruses with comparably low human pathogenic potential. In further studies, the seasonal variation of the virome will be analyzed to identify possible shedding patterns for particular viruses.

6. Discussion

The following chapter is divided into three sections and based on the underlying aims of this thesis. First, the establishment of the sampling strategy will be discussed in the context of the comprehensive study (see chapter 6.1). Second, the obtained results and characterization of novel virus strains will be discussed in the context of the different detection methods applied (see chapter 6.2). Third, the results will be assessed regarding their relevance for public health and epidemiological issues (see chapter 6.3).

6.1 Bat sampling and establishment of a comprehensive sampling strategy

This thesis aimed to trap and sample bats in Wavul Galge cave, Sri Lanka, in order to identify prevalent viruses in different bat species. In addition, the collected samples were intended to be used for other research questions in the context of zoology, ecology and microbiology (see chapter 4.3). Therefore, it was of high importance to develop a mutual sampling strategy meeting the requirements of all represented research fields. At the same time, the high expertise of involved researchers with different specializations was beneficial for designing the implemented pilot study.

For this purpose, the general conditions of the sampling sessions were trialed and optimized. Protective measures influenced the strategy of how to catch, handle and sample bats. Different bat samplings in morning and evening sessions were conducted and compared, the maximum number of bats sampled per session was tested and the types of samples and associated documentation per bat were determined. Examples of the trapping and sampling of bats are visualized in Figure 5.

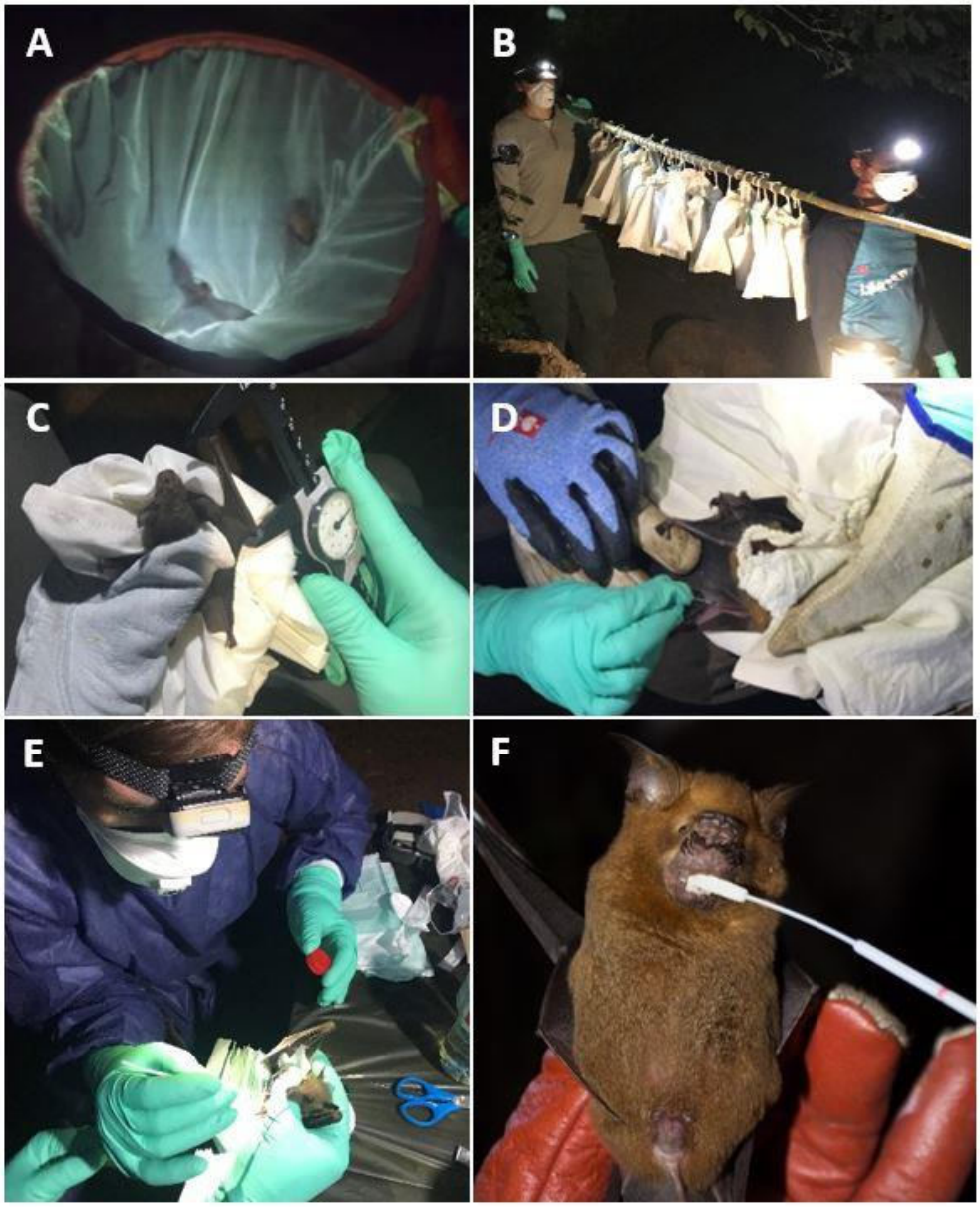

Figure 5: Impressions of the bat samplings conducted in Wavul Galge cave in Sri Lanka. A: Catching of bats by using a hand net. B: Bats are kept in holding bags and transported to the field station for sampling. C: Taking forearm measurements from an *R. rouxii* bat for documentation. D: Taking blood samples of an *H. speoris* bat by venepuncture. E: Taking an oral swab sample from an *R. leschenaultii* bat. F: Taking an oral swab sample from an *H. speoris* bat. © Sahan Siriwardana

In this process of optimization, the focus was on a wide spectrum of different research questions. Priority in terms of zoology was the research on the different bat species inhabiting the Wavul Galge cave. Sex, weight and forearm length of each bat were recorded as characteristic measurements. Health condition and age of each bat were estimated, which will allow to assess the overall condition of the bat colony inhabiting Wavul Galge cave. The

Cytochrome B sequencing of bat oral swab samples was implemented to determine accurately the present bat species [190]. With these results, previous macroscopic species determination was confirmed with the help of molecular methods. Such data will serve as groundwork for further systematic studies on the bat population of Wavul Galge cave.

In close connection to this, ecological questions were aimed to be resolved with the comprehensive sampling strategy. Wavul Galge cave harbors the bat species *M. fuliginosus*, *R. rouxii*, *R. leschenaultii*, *H. speoris* and *H. lankadiva* (see chapter 3.1.6). A summary of important characteristic features per species is given in Table 3.

Table 3: Overview of some ecological characteristics of the five bat species inhabiting Wavul Galge cave, Sri Lanka, as observed or confirmed during the three bat sampling sessions in March 2018, July 2018 and January 2019 [191].

	Hipposideros lankadiva	***Hipposideros speoris***	***Miniopterus fuliginosus***	***Rousettus leschenaultii***	***Rhinolophus rouxii***
Nutrition	Insectivorous	Insectivorous	Insectivorous	Frugivorous	Insectivorous
Outflight order	2nd	2nd	1st	3rd	1st
Reproduction cycles	March – May	April – May, September – October	July – August	April, September	April – May, September – October
Migration	No	No	Yes	No	Yes

The correlation of roosting and migrating behavior of all bat species in this sympatric colony is of interest to improve the general ecological knowledge.

For example, except for the fruit bat *R. leschenaultii* all other species inhabiting Wavul Galge cave are insectivorous bats. Therefore, comparative nutrition analyses are planned to be conducted with the samples (compare Figure 4) and were included as part of the comprehensive sampling strategy. Analyzing feces samples for cytochrome c oxidase I (COI) can help to differentiate the consumed invertebrates and to understand the feeding behavior, possible foraging competition between the bat species and the general contribution of bats to important agriculture and ecosystem services [192].

The conducted sampling sessions revealed the need for future bat counting in order to better estimate the population size and species composition of this colony. For example, the second sampling took place in July 2018 and represented a population peak for *M. fuliginosus* in Wavul Galge as pregnant bats migrated to this cave before parturition. As a result, bats of this species

were overrepresented during this sampling session. With the knowledge of such population peaks and other basic parameters of the sympatric bat species (Table 3), future sampling sessions can be planned accordingly.

Furthermore, the roosting dynamics and interaction of sympatric species inside the cave are aimed to be investigated. Previous observations in Wavul Galge have identified specific and separated roosting locations inside the cave for each species (Figure 6). Close contact between the present species is observed when the nocturnal animals leave the cave in the evening and return in the morning. But even this process is predetermined, follows a specific order by bat species and further separates them (see Table 3).

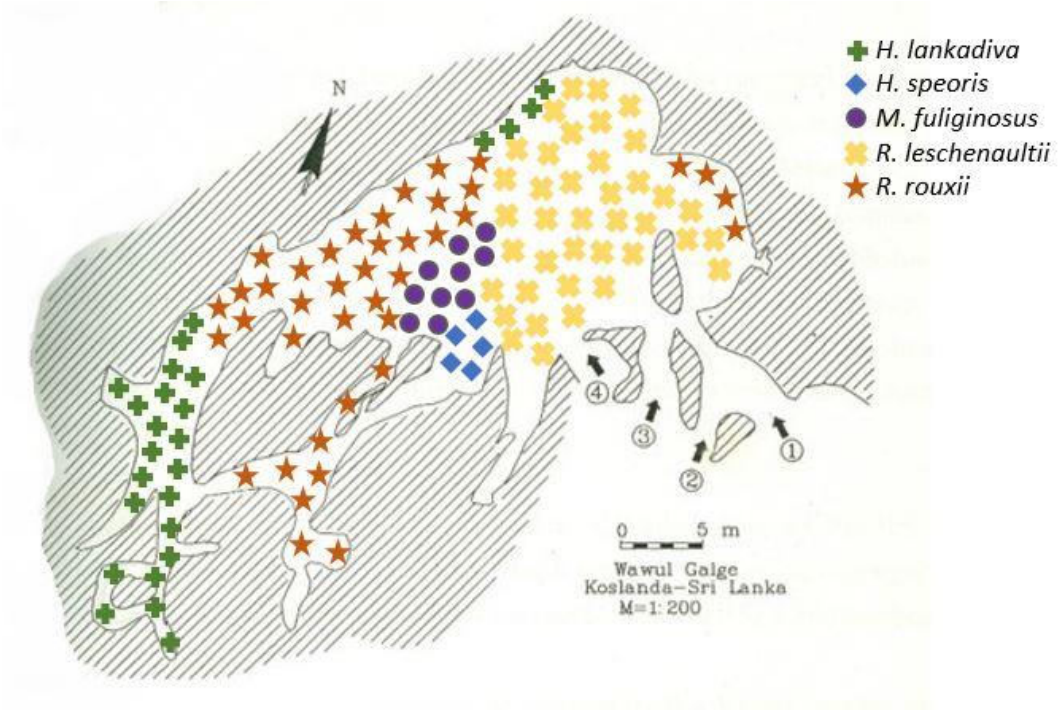

Figure 6: Schematic plan of Wavul Galge cave, specifying the roosting places of the different bat species and the entrances (1-4) to the cave (© Wipula Yapa).

In terms of microbiological and virological research, thorough planning of the sampling strategy was a prerequisite. In search of viruses and other microorganisms, mainly nucleic acid-based detection methods were used which will be discussed in detail in the following section (see section 6.2). For this purpose, intact nucleic acids are required. Also, further envisaged virus detection methods such as electron microscopy and cell culture require a good sample quality including intact infectious virus particles. This can be achieved by proper sampling and

sample logistics. Storing the samples at low temperatures is ideal to preserve the microbial content as far as possible [193]. Multiple freeze-and-thaw cycles affect virus integrity and should be avoided. Sri Lanka is a tropical country with average temperatures of 27 °C in a range of 22 °C to 31 °C. Especially in the field and sampling setup the cooling options are limited and maintaining a proper cold chain is challenging. Cooling boxes are not sufficient to keep the samples frozen for several days due to the high ambient temperatures. The ideal solution is the sample storage using a dry shipper, a liquid nitrogen vapor tank cooling the samples at -196 °C for up to 2 weeks [194]. This represents an approved and safe possibility for good quality sample storage even of potentially infectious samples according to the regulations of the International Air Transport Association.
Additional preservation can be achieved by storing the samples in specific transport media or liquids such as PBS, although this may hamper the usability of the sample for the broad range of envisaged analyses [195].

In the conducted samplings, samples were therefore snap-frozen and transported without any liquids, and the further sample processing was realized afterwards in the laboratory. According to the results included in this thesis, the dry storage of samples did generally not impede the outcome of virological analyses as diverse viruses were detected via PCR and metagenomic NGS (see sections 5.3 to 5.6). In the course of other research conducted, the analysis of the bat microbiome was also possible with these samples. However, the applied detection methods can not reveal if other less stable viruses were possibly degraded during the storage and transportation. The virus integrity of the samples remains to be tested; virus isolation in cell culture systems will be attempted with the samples tested positive (see section 7).

In future sampling sessions, multiple samples in different suitable transport media may be taken per bat if necessary for an improved result outcome. However, this would considerably increase the workload and the number of samples to be stored. In conclusion, taking one sample per bat and sample type that may be processed and used simultaneously for multiple questions, as was done in the pilot study, will be beneficial for the long-term sampling strategy.

In this context, also the specific strategy for taking and storing blood samples was involved. Instead of storing collected blood drops in vials, blood was collected on protein saver cards and could be stored in dry format at room temperature. This facilitated the storage and transport of this sample type, while the quality remains to be tested but is assumed not to be affected [196].

Concluding Statement: Bat sampling

- Three bat samplings were conducted in Wavul Galge cave, Sri Lanka
- Different sampling time points aim to cover seasonal changes in the population
- Different sample types were collected to resolve questions from the fields of ecology, zoology, virology and microbiology
- A comprehensive sampling strategy was developed for future bat samplings in the cave and other locations

6.2 Analysis of bat samples using different virus detection methods

The sampling of bats in Wavul Galge cave, Sri Lanka, was followed by the analysis of selected samples with different methods as described in chapter 4.3 (see Figure 4). The following Table 4 gives a more detailed overview of the collected samples during all three sampling sessions per species. Furthermore, it is indicated from which collected samples the results of this thesis were obtained.

Table 4: Overview of the three sampling sessions conducted in the framework of this thesis, including number of collected samples per bat genus. Adapted from: [191]. The roman numerals indicate from which sample the respective results of this thesis were obtained (Publications II – V, see chapter 5).

		Oral swabs	**Rectal swabs**	**Feces**	**Urine swabs**	**Blood**	**Ectoparasites**
***Hipposideros* spp.**	**Total (bats)**						
03/2018	**3**	3	3 III	0	2 II	0	0
07/2018	**1**	1	0	0	0	0	0
01/2019	**22**	22	16 III	7 III	6 II	21	3
M. fuliginosus	**Total (bats)**						
03/2018	**3**	3	3 III	0	0	0	2
07/2018	**188**	188 V	116 III, IV	77 III, V	102 II, V	10	10
01/2019	**31**	31	4 III	27 III	11 II	23	10
R. leschenaultii	**Total (bats)**						
03/2018	**9**	9	9 III	2 III	2 II	1	5
07/2018	**11**	11	11 III	0 III	2 II	3	5
01/2019	**20**	20	16 III	3 III	6 II	18	10
R. rouxii	**Total (bats)**						
03/2018	**67**	67	60 III	8 III	6 II	0	10
07/2018	**0**	0	0	0	0	0	0
01/2019	**34**	34	16 III	17 III	6 II	23	10

As indicated in Table 4, this thesis included the analysis of oral swab, rectal swab, fecal and urine swab samples from different bat species and sampling sessions. With slight differences depending on the sample type, all samples were processed and nucleic acids were extracted. In the general procedure (overview see Figure 4), PCR screenings for particular viruses of interest (see chapter 6.2.1) were performed with the individual nucleic acid extracts. For NGS analyses (see chapters 6.2.3 and 6.2.2), samples belonging to the same sampling session, bat species and sample types were pooled, respectively. The obtained results will be discussed in the following chapters 6.2.1 to 6.2.2.

6.2.1 PCR screening for particular viruses of interest

In the course of this thesis, different virus detection methods were applied, and identified viruses were characterized thoroughly based on the available data.

With the application of generic PCR assays, all samples were screened for particular viral families that have already been reported in these bat species, but in different locations worldwide. As part of this thesis, the samples were screened for the presence of different viruses, and the respective sample types to test were selected depending on the typical shedding route. Feces and rectal swab samples were screened for *Coronaviruses* [197] and *Adenoviruses* [198], urine samples for *Paramyxoviruses* [199] and oral swab samples for *Lyssaviruses* [200]. Furthermore, due to the concrete suspicion of bat *Filoviruses* in *R. leschenaultii* as previously reported in China in 2019 [201], rectal swab samples and oral swabs of this species were also screened with a family-specific PCR for the detection of *Filoviruses* [202].

From all above-mentioned family-specific PCRs applied in this study, only the screening for CoV and PMV revealed positive results which were also published in the framework of this thesis. All PCRs were carried out using suitable controls, furthermore the sample integrity was verified in the subsequent NGS analysis. A summary of the obtained PCR results is given in Table 5 [203, 204].

Table 5: Overview of the CoV and PMV PCR screening results included in this thesis. The results are differentiated according to sample type and bat species. The number of samples tested positive and total sample number (all sampling sessions) are given. Tables are adapted from Muzeniek et al., 2021 [203] and Muzeniek et al., 2022 [204]

	CoV Screening		**PMV Screening**
	Rectal Swabs	Feces	Urine swabs
***Hipposideros* spp.**	0/20	0/8	0/8
M. fuliginosus	20/122	11/103	10/113
R. leschenaultii	2/35	0/5	0/10
R. rouxii	0/78	0/26	0/12

The generic PCR approach is beneficial as the presumed presence of particular viruses can be directly and easily proved with PCR screening, provided the viral load is not below the detection limit of the assay. Generic PCRs often produce an amplicon size of several hundred base pairs (bp) and allow for subsequent Sanger sequencing of the product. Furthermore, the assays are

commonly designed on a conserved genome region to detect all representatives of the viral family [205]. With this approach, analysis of these identified virus strains was possible by using the obtained Sanger sequencing data.

For *Coronaviruses*, it was possible to differentiate between the presence of an α-CoV in *M. fuliginosus* bats and a β-CoV in *R. leschenaultii* bats. A phylogenetic tree of the available sequence on a highly conserved gene allowed for a tentative assessment of the pathogenic potential of both identified CoVs.

Furthermore, the screening for PMVs revealed the presence of a bat *Paramyxovirus* in *M. fuliginosus* bats, and further Sanger sequence analysis indicated the persistence of several PMV strains and allowed for tentative phylogenetic calculations.

However, even in family-specific PCR assays the genericity is limited and assay design requires minimum knowledge of the primer binding sequences. A more open detection and unbiased view is possible with advanced molecular technologies such as the NGS analyses conducted.

Concluding Statement: PCR screening

- Different sample types were screened for particular viruses of interest using generic PCR assays
- Urine swab sample of the bat species *M. fuliginosus* were tested positive for PMV
- Sequence analysis and phylogeny indicate the persistence of three PMV strains in *M. fuliginosus*
- Rectal swabs and feces samples of the bat species *M. fuliginosus* and *R. leschenaultii* were tested positive for CoV
- Sequence analysis and phylogeny reveal the presence of α-CoV in *M. fuliginosus* and β-CoV in *R. leschenaultii*

6.2.2 Full genome assembly and virus characterization

In this thesis, bat samples were sequenced by using HiSeq 2500 or NextSeq 500 instruments (Illumina, San Diego, CA, USA) which are common platforms for the high throughput sequencing of virus genomes and viromes [206]. The general workflow of metagenomic sequencing and data analysis as performed in this thesis is illustrated in the following Figure 7.

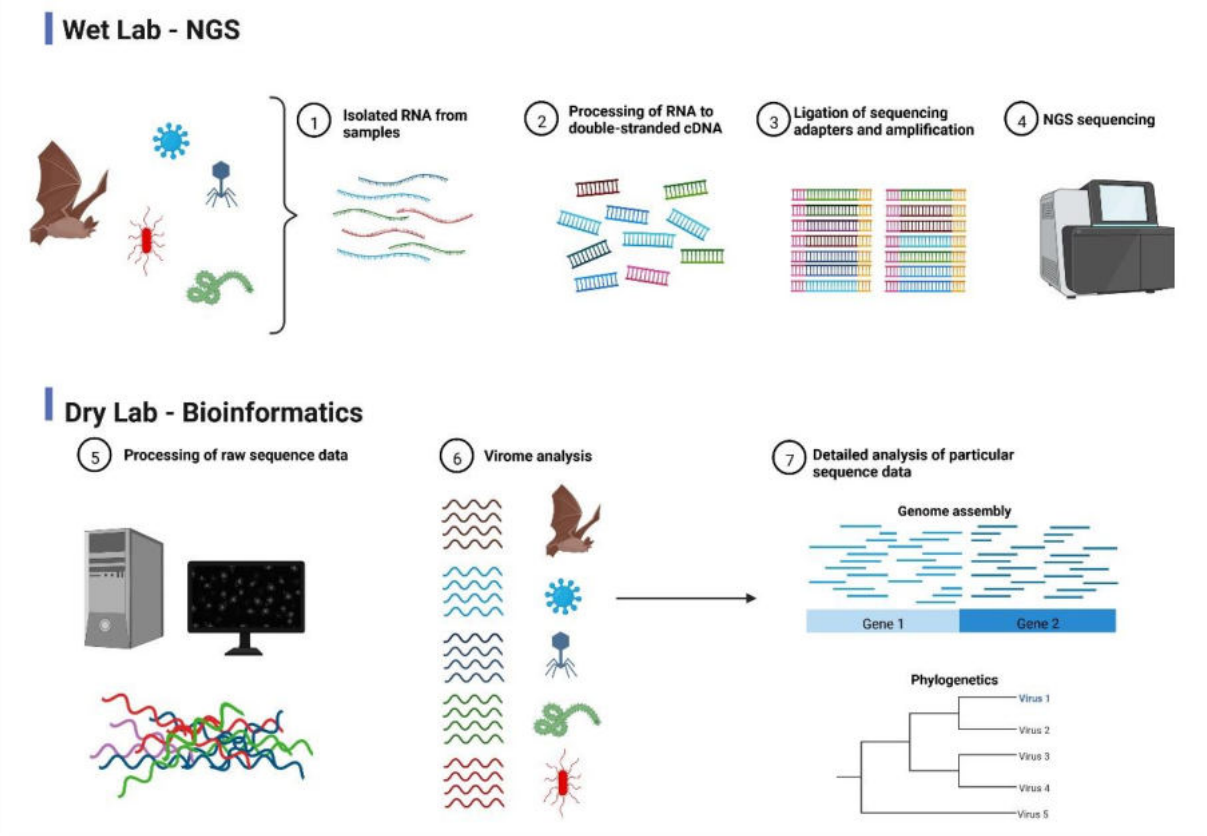

Figure 7: Schematic illustration of the general NGS workflow (wet lab) with subsequent bioinformatic analysis of obtained data (dry lab). The goal is to identify and further characterize novel viruses from the bat samples [207]. Created with BioRender.com

As shown in the workflow (Figure 7), the NGS analysis may be divided into the wet lab part that includes the sample preparation and the dry lab part that includes bioinformatics and sequence data analysis. The sequencing procedure applied in this work provides an open view and every nucleic acid can be sequenced, including genomic sequences of the host and from other sources such as bacteria or fungi [208]. Depending on the available NGS data, also full genome assemblies of particular viruses can be achieved by bioinformatic analysis.

Part of this thesis was the assembly of a bat α-CoV full genome from *M. fuliginosus* rectal swab samples [209]. The following considerations in this section will therefore mainly focus on the analysis and results obtained from the full genome sequencing and virus characterization of the novel α-CoV strain batCoV/MinFul/2018/SriLanka.

A large proportion of sequencing data for the full genome analysis was revealed from an NGS data set; with its unbiased approach large parts of the genome were amplified and sequenced. Nevertheless, parts of the genomic sequence data were missing or did not meet the quality criteria (e.g. insufficient read coverage, sequence diversity of matching reads). For these cases, specific PCR assays were designed based on the available data to cover the gaps. The full genome was in large part assembled from NGS data and completed with Sanger sequences from the spanning PCR assays. The full genome sequence enabled a comprehensive analysis and in-depth characterization including the analysis of viral relationships, which is not or only to some extent possible with partial sequences [210, 211]. The assembly of the full genomic sequence allowed for the precise detection of open reading frames and subsequent annotation of genes. Even for virus species belonging to the same viral family, the length of particular genes may vary to some extent. For example, members of the family *Coronaviridae* have the longest genomes among all RNA viruses, ranging in size between 26 and 32 kb [106]. Differences in the length and sequence of particular genes can result in altered folding of proteins, which can in turn have an impact on infectivity, host specificity, transmissibility and other factors [106]. Therefore, it is of high interest for virus research to obtain sequence data as complete as possible for the subsequent characterization.

For the bat α-CoV that was analyzed in the course of this thesis, the characteristic genes of CoVs were identified. This included the ORF1a/b polyprotein and encoding non-structural and functional proteins including the RNA-dependent RNA Polymerase (RdRP). Furthermore, ORFs for the structural proteins for spike, envelope, membrane and nucleocapsid proteins were allocated to the full genome sequence and were similar to the generally known genome organization of CoVs. Additional ORFs are present in different numbers depending on the respective CoV species and subfamily, which also results in the variable genome sizes of CoVs [212]. However, in the course of this work, the annotation focused on the generally recognized genes of all CoV genomes which are depicted in Figure 8.

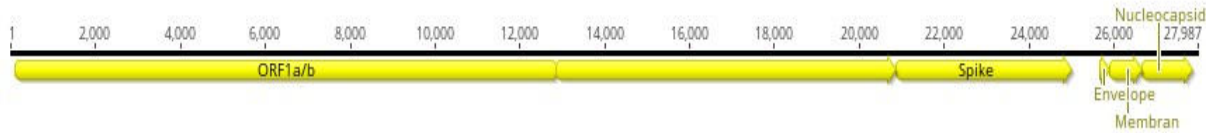

Figure 8: Genome organization of the novel α-CoV strain batCoV/MinFul/2018/SriLanka (OL956935) using the annotation tool in Geneious Prime® software (version 2021.2.2).

For further sequence and phylogenetic analyses, the differences in particular highly variable or conserved genes are important to consider.

DNA viruses commonly use available DNA polymerases including proofreading activity in the host cell; therefore they are generally less susceptible to mutations. This employment of host enzymes is not possible for RNA viruses which are generally susceptible to high mutation rates of between 10^{-6} to 10^{-4} substitutions per nucleotide per replication cycle [213].

The only exception are some members of the order *Nidovirales*, including the family of *Coronaviridae*, that have an RNA polymerase including 3'- to 5'- exonuclease activity [214]. With this, the mutation rate is rather comparable to the rates of small ssDNA viruses and about 10-fold lower compared to that of influenza virus [215, 216]. Nevertheless, the long-term mutational changes in CoVs appear to be comparable to that of other RNA viruses and may be a result of their large genome size and high replication rates in the host cells [214].

A common model for understanding the genetic evolution of viruses and consequences of high mutation rates is the quasispecies theory which was initially described by Eigen in 1971 [217]. In its original form, the theory describes that from a master copy of the viral RNA sequence a number of mutant copies are produced with a certain probability distribution and as a function of the nucleotide substitution rates during the replication process [218]. This pool of copies is also described as "mutant spectrum" or quasispecies and represents the variety of viral sequences during replication in the host cell. In the context of virus evolution, these quasispecies provide the basis for negative or positive selection of particular mutations or viral variants [219]. From the pool of viral sequence copies in the host cell, those genotypes with beneficial phenotypical traits will become dominant. In contrast, negative selection applies to those genotypes which have no or less functional phenotypes due to the mutations, resulting in their elimination from the quasispecies pool.

Therefore, the establishment of successful mutations and resulting changes in amino acid sequence to some extent depends on the function of the respective gene or ORF and the corresponding function of the protein. For example, the non-structural genes such as RdRP located on the ORF1b of CoVs are required to fulfill their function in replication of the virus genome. Mutations in these genes may lead to loss of function of the protein and elimination of these genotypes in the context of negative selection. Therefore, these non-structural genes are generally considered less susceptible to mutations [131, 220]. Furthermore, the genes for membrane and envelope proteins evolve slowly with comparably low mutation rates [221].

These conserved regions are often used for phylogenetic analysis, the phylogeny of the RdRP is considered as standard method as this highly conserved gene also allows for the comparative analysis between different viral families, for instance those of the same viral order [222]. Conserved regions are also often used for the design of family-specific PCRs like those used for the successful screening of CoV and PMV in this thesis [203, 204]. As already discussed in the corresponding section (see section 6.2.1), the PCR amplification and comparison of partial RdRP sequences already allowed for a preliminary characterization of the detected virus.
An example of a phylogenetic reconstruction based on the partial RdRP gene of the CoV genome is given in Figure 9 [203].

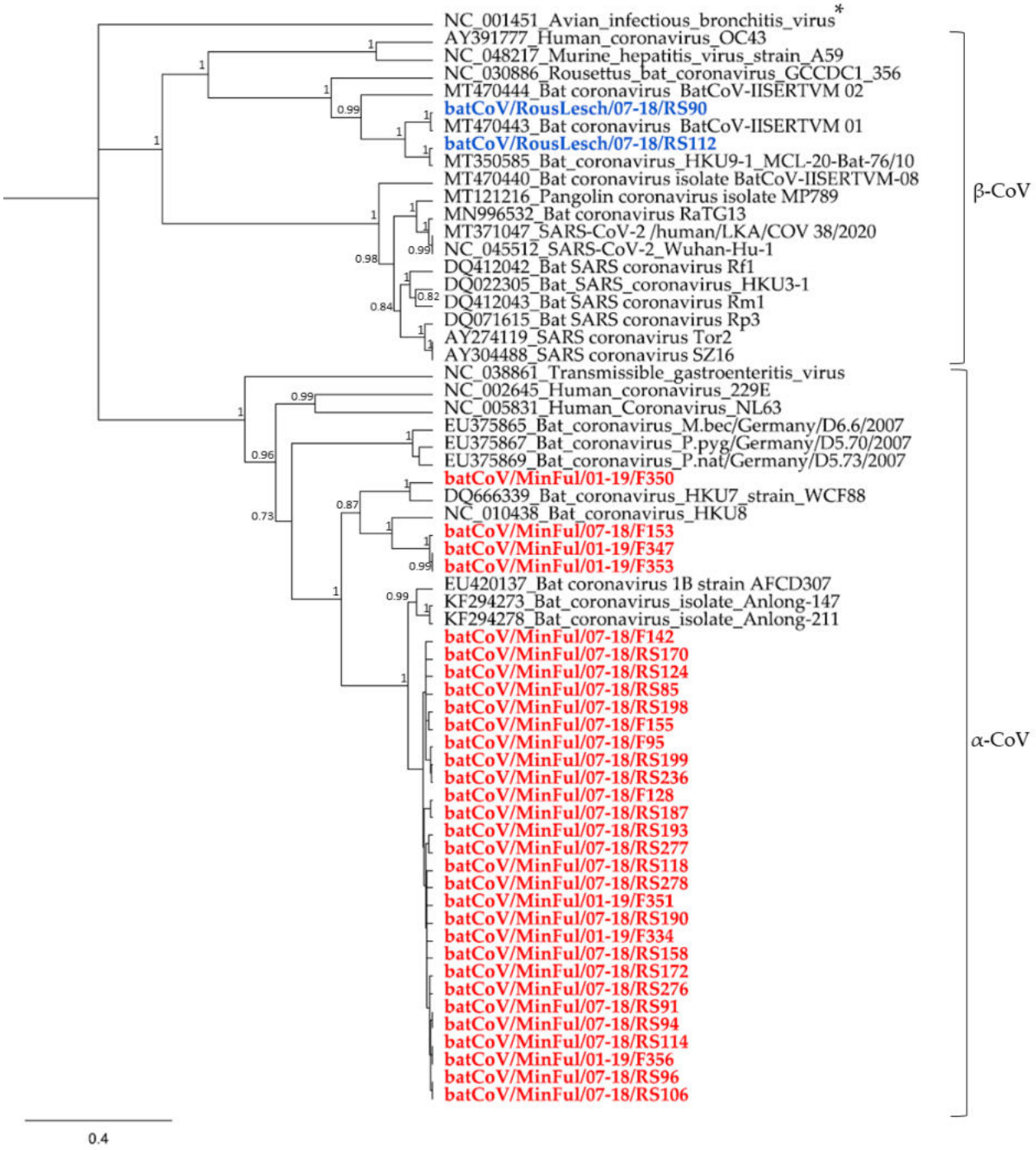

Figure 9: Example of a phylogenetic reconstruction of coronavirus sequences from Sri Lankan *M. fuliginosus* bats (given in red) and *R. leschenaultii* bats (given in blue) and other reference sequences of different α- and β-CoVs. Based on the partial RdRP sequence used for this reconstruction, the tree is divided into the two groups of α- and β-CoVs. In addition, the γ-CoV avian infectious bronchitis virus (NC_001451, marked with an asterisk) was included as outgroup for the calculation [203].

However, with the sequencing and comparative analysis of the complete RdRP gene or rather ORF1b polyprotein, a comprehensive analysis of the gene was possible. In the case of CoV as well as many other virus families, the characterization of the complete RdRP gene is also the species demarcation criterion according to the ICTV [223]. For CoV, the complete genome sequence needs to be available for taxonomic evaluation. Nevertheless, the species demarcation is only based on the RdRP gene, and a sequence identity of 90 % or less on aa level to other virus species of the family is the prerequisite to be recognized as novel species [101]. The comparative analysis of the detected α-CoV in this thesis calculated identities of 94.9 % (ORF1b, aa level) to the closely related Miniopterus bat coronavirus 1 (Acc. EU420138), an α-CoV species recognized by ICTV [209]. Consequently, the detected CoV in this thesis may be considered rather as a novel strain of this virus species within the genus *Minunacovirus* [209]. The phylogenetic analysis also revealed close relationships of α-CoVs derived from the host genus of *Miniopterus* bats and the clear distinction to other subgenera and human pathogenic CoV such as SARS-CoV, SARS-CoV-2 as well as the α-CoVs HCoV-229E and HCoV-NL63. In this context, the phylogenetic analysis also allowed to comprehend the genetic evolution of the family of *Coronaviruses*. The evolution of different CoV subgenera can be retraced by their separation into different branches of the phylogenetic tree. In this context, it is important to consider that the selection of CoV species for phylogenetic calculation can have an impact on the outcome. The selection of a high variety of CoVs from different subgenera allowed for retracing the evolutionary relationships as described. However, the selection of multiple virus species within the α-CoV genus or virus strains of the virus species of interest can reveal in-depth relationships within this particular virus species of interest. The complexity of analysis and selection of strains is therefore highly dependent on the respective question and desired statement of phylogenetic analysis.

In this thesis, the phylogenetic analysis of the newly detected α-CoV was calculated with a selection of different α-CoVs and β-CoVs representing the respective subgenera and enabling the rough classification and tracing evolutionary relationships. The inclusion of a higher number of representative strains from the subgenus *Minunacovirus* and the *Miniopterus*-borne α-CoVs allowed for the reliable allocation of the novel virus strain into this subgenus and to the virus species Miniopterus bat coronavirus 1 [209].

Analyzing geographical relationships of viruses can be interesting for understanding the distribution of the virus by the migrating host species *M. fuliginosus*. This was impeded because only sparse sequence data were available from nearby countries such as India. A bias of available sequence data was generally observed as a high number of relevant data was obtained from submissions from China [105, 224–231]. Supposedly this high number of CoV-related entries is a result of biased research on CoVs and their bat hosts after the emergence of SARS-CoV in 2002, as already discussed earlier [232].

However, the in-depth analysis of other genes was also of interest for the extensive characterization of the newly detected α-CoV. While the comparison of conserved genes mainly represents evolutionary relationships between the virus species, the analysis of highly variable gene regions can reveal information about the virulence of viruses and the influence of mutations in these sequence regions.

The selective pressure on other genes can be higher than in non-structural genes, and structural components of the virus particle such as the spike protein in CoVs show more successful mutations and may benefit from positive evolution [221]. This is usually a response to host adaptation as mutations may change the virulence and transmissibility of the virus or help to escape the immune system of the host [222].

Therefore, the sequence and phylogenetic analysis of the highly variable spike protein and other common substitution patterns can help to decipher the genetic changes and evolution of a virus caused by selective pressure, and it is therefore another important analysis tool in disease epidemiology as it was also commonly used during the Covid-19 pandemic [222].

Since the emergence of SARS-CoV-2 prominent examples are the point mutations primarily located in the spike protein, resulting in the development of new variants with altered characteristics regarding infectivity, transmissibility and severity of disease [233]. The spike protein is part of the virus particle and responsible for the attachment to host cells and subsequent endocytosis. Mutations and accompanying alterations in protein folding can enhance the binding efficiency to the receptors and improve the entrance into the host cells [233].

The analysis of specific sequence motifs can reveal information about the pathogenic potential and host-specificity of the investigated virus. This is especially important when characterizing

a novel virus strain as in the context of this work. For CoV, one essential receptor binding domain is located on the S1 subunit of the spike protein and responsible for recognition of and binding to the host cell. The RBD of SARS-CoV-2 binds to the ACE2 receptors that are present on human lungs, kidney, heart and intestine cells [234]. Additionally, the S1 subunit of human pathogenic CoVs contains a furin cleavage site motif, further enhancing the entrance effectivity of the virus into the human host cell [235].

For analyzing such sequence motifs an MSA of the respective gene section of interest was calculated with a number of α-CoVs and β-CoV as references. In-depth sequence comparison revealed the presence of these particular motifs only in the human pathogenic strains. Its absence in the novel α-CoV strain from Sri Lanka represented another characterization of this newly detected virus strain and supports the assumption of a low human pathogenic potential.

Concluding Statement: CoV genome assembly and characterization

- A CoV full genome was assembled from mNGS data and PCRs of *M. fuliginosus* rectal swabs collected in July 2018
- The novel strain is highly similar to Miniopterus bat coronavirus 1, a virus species within the genus *Minunacovirus*
- Genes were annotated and further analyzed based on in-depth sequence analysis and phylogenetic reconstruction
- Analysis of different sequence motifs in the spike protein indicates a rather low human pathogenic potential of the novel CoV strain

6.2.3 Analysis of samples using metagenomic NGS

The general metagenomic workflow as applied in this thesis was already described in chapter 6.2.2 (see Figure 7). Apart from the full genome assembly of particular viruses of interest, this unbiased method is also suitable to discover the general virome composition in a sample with an open view approach. Although the unbiased sequencing is a general advantage of mNGS, the presence of other non-viral background sequences may impede the discovery of the targets of interest, such as the detection of viral sequences in this work. Especially in feces samples, bacterial sequences of the microbiome are likely to be overrepresented. A digestion of background DNA was included in the workflow to allow for the more targeted detection of RNA viruses that were of highest interest in this study. The majority of known human pathogenic viruses, including those of zoonotic origin, are RNA viruses [40, 236]. Therefore, even open view methods such as NGS may have an intended bias as the bat virome is often analyzed with special focus on particular viruses that are currently of interest. Such bias was often reported after outbreak situations, such as SARS-CoV, SARS-CoV-2, Hendra and Nipah virus or Ebola virus outbreaks. After the emergence of these viruses, an increased number of studies was performed on the suspected reservoir animals, e.g. *Rhinolophidae* bat species as hosts for SARS-CoV [232, 237], *Pteropodidae* bat species as hosts for Hendra and Nipah viruses [238, 239] and different fruit bat species as hosts for Ebola viruses [240]. Consequently, the analysis of such mNGS data in respective studies is often performed with a focus on particular virus families or groups.

In this thesis, mNGS data analysis was focused on the detection of RNA viruses. The applied sample preparation protocol included an initial digestion of DNA to reduce the number of genomic sequences of the bat host and to increase the probability of detecting the viral sequences of interest. This consequently implied a bias of the virome towards RNA viruses.

With the generated sequence data sets of this pilot study, a variety of metagenomic analysis and comparisons with different output can be applied. Compared to the previously described detection approach of PCR screening, metagenomic NGS allows for the detection of a variety of viruses and possible pathogens in the samples by using one particular workflow. This is especially useful for the detection of uncommon or yet unknown viruses [241].
Part of this thesis was the comparative virome analysis of different sample types collected from *M. fuliginosus* bats at one sampling time point. The analysis of the mNGS data was achieved by matching the obtained reads against the current viral protein database obtained from NCBI (compare Figure 7).

The comparison of mNGS data from oral swabs and feces and urine samples revealed different viral compositions. All sample types contained a high number of sequencing reads assigned to *Microviridae*. This genus represents ssDNA phages that are known to infect bacteria (bacteriophages) [242]. In terms of human pathogenic viruses, this family is of rather low relevance, although it indicates the wide spectrum of bacteria that can also be found in bats. In combination with targeted 16S sequencing of the samples, these data can reveal valuable information about the bats' microbiome and support the comprehensive study of the bats.

Sequencing reads for PMVs as well as CoVs were identified in urine and feces samples, as they were already tested via PCR screening (see chapter 6.2.1). In addition, a number of other viral sequences were identified in different sample types, such as *Picornaviridae*, *Iflaviridae* and unclassified *Riboviria* in feces, and furthermore different *Astroviridae* in feces and urine samples [207]. A summary of these results is given in Table 6.

Table 6: Overview of the results obtained from mNGS data analysis of feces and urine swab samples from *M. fuliginosus* bats collected in July 2018. The table indicates the related virus, sample types and pool numbers from which the results were obtained, including the number of assigned reads, longest contig and nucleotide identity. The name and accession number of the novel virus strain as uploaded to GenBank are indicated, respectively [207].

	Astroviridae	
Name of related virus	**Bat astrovirus 1 isolate AFCD77 (EU847151)**	
Sample type	Feces samples	Urine swab samples
Pool numbers	F1, F2, F3, F4, F5, F6, F7, F8	U4, U8, U9, U14, U15
Assigned reads / longest contig	289 / 1068 nt	422 / 379 nt
Nucleotide identity	82 %	85.6 %
Name of novel virus strain	Bat astrovirus isolate F2/18 (Acc. OP141159)	Bat astrovirus isolate US2/18 (Acc. OP141166)
Name of related virus	**Mamastrovirus 14 isolate AFCD57 (NC_043099)**	
Sample type	Feces samples	
Pool numbers	F3	
Assigned reads / longest contig	1045 / 1366 nt	
Nucleotide identity	86.5 %	
Name of novel virus strain	Mamastrovirus 14 isolate F2/18 (Acc. OP141160)	
Name of related virus	**Mamastrovirus 18 isolate AFCD337 (NC_043102)**	
Sample type	Urine swab samples	
Pool numbers	U3, U5, U6, U9, U11, U12, U14	
Assigned reads / longest contig	282 / 311 nt	
Nucleotide identity	84.4 %	
Name of novel virus strain	Mamastrovirus 18 isolate US2/18 (Acc. OP141167)	
	Coronaviridae	
Name of related virus	**Bat alphacoronavirus isolate batCoV/MinFul/2018/SriLanka (OL956935)**	
Sample type	Feces samples	Urine swab samples
Pool numbers	F1, F2, F3, F4, F5, F6, F7, F8	U2, U3, U4, U5, U6, U8, U9, U12, U13, U 14
Assigned reads / longest contig	994 / 729 nt	10,753 / 1226 nt
Nucleotide identity	83.1 %	98.6 %
Name of novel virus strain	Bat alphacoronavirus isolate MinFul/F2/2018/SriLanka (Acc. OP141161)	Bat alphacoronavirus isolate MinFul/U2/2018/SriLanka (Acc. OP141168)
Name of related virus	**BtMf-AlphaCoV/GD2012 (KJ473797)**	
Sample type	Feces samples	Urine swab samples
Pool numbers	F1, F2, F3, F4, F5, F7	U4, U6, U9, U12, U13, U14
Assigned reads / longest contig	2443 / 1113 nt	2182 / 769 nt
Nucleotide identity	83.1 %	85.5 %
Name of novel virus strain	Bat alphacoronavirus isolate AlphaCoV/F2/2018 (Acc. OP141162)	Bat alphacoronavirus isolate AlphaCoV/U2/2018 (Acc. OP141169)
	Iflaviridae	
Name of related virus	**Spodoptera exigua iflavirus 2 isolate Korean (JN870848)**	
Sample type	Feces samples	
Pool numbers	F1	
Assigned reads / longest contig	373 / 1283 nt	
Nucleotide identity	96.8 %	
Name of novel virus strain	Spodoptera exigua iflavirus isolate F2/18 (Acc. OP141163)	

	Paramyxoviridae
Name of related virus	**Jingmen Miniopterus schreibersii paramyxovirus 1 (MZ328288)**
Sample type	Urine swab samples
Pool numbers	U2, U3, U6, U7, U9, U11, U12, U14
Assigned reads / longest contig	573 / 450 nt
Nucleotide identity	84 %
Name of novel virus strain	Miniopterus schreibersii paramyxovirus isolate US2/18 (Acc. OP141170)
	Picornaviridae
Name of related virus	**Miniopterus schreibersii picornavirus 1 (JQ814851)**
Sample type	Feces samples
Pool numbers	F1, F2, F3, F4, F5, F6, F7, F8
Assigned reads / longest contig	5714 / 3452 nt
Nucleotide identity	86 %
Name of novel virus strain	Miniopterus schreibersii picornavirus isolate F2/18 (Acc. OP141164)
	Unclassified Riboviria
Name of related virus	**Hubei sobemo-like virus 21 strain CC64469 (KX882813)**
Sample type	Feces samples
Pool numbers	F1, F3, F4
Assigned reads / longest contig	439 / 534 nt
Nucleotide identity	82.1 %
Name of novel virus strain	Hubei sobemo-like virus isolate F2/18 (Acc. OP141165)

Most of the detected viruses described in Table 6 are commonly found in viromes of the bat genus *Miniopterus*, and the results also correspond with the typical shedding routes that were reported for closely related viruses [231, 243–245]. Almost complete viral genes and genomes were furthermore assembled from the NGS data of urine and feces, matching rather unexpected viruses such as Iflavirus and Hubei-sobemo-like virus. These viruses were originally sequenced from insect or worm samples. Detected in feces samples, such viruses most probably originate from the nutrition of the bats [246, 247]. With the available sequence data from only non-invasive samples it is not possible to state whether the bats were actually infected with the virus or whether sequences were solely obtained from the digested insects. To detect viral infections, different bat organs would be necessary to analyze, but these were not available from the conducted non-invasive samplings. However, this kind of obtained sequence data can support preliminary evidence on the diet of this bat species. The data may be synchronized and compared with future sequencing data obtained from targeted nutrition analysis.

The viral sequences identified in the course of this thesis shared identities between 80.36 % and 98.37 % to the closest related viruses based on the available sequence data [207]. Further sequence data will be necessary to conclude whether the present viruses are either novel virus species or strains of an already known virus species. This depends on the respective virus family and the associated ICTV species demarcation criteria.

An estimated proportion of 50 to 70 % of mNGS data show no similarities to available viral sequences from GenBank [248]. Probably such sequences may represent background, host or bacterial sequences as discussed before. Similarly, it is possible that viruses are present in the sample for which the sequences are yet completely unknown and therefore not comparable to the available databases. On the basis of these estimates, the extent of yet unknown and unexplored viruses and the actual virus species richness are presumed to be vast. The increasing investigation of whole viromes and extension of viral databases help to successively explore the viral richness in a diverse range of human, animal and environmental samples. In the case of viral outbreaks, such extensive databases can support the monitoring and surveillance of the outbreak and a faster risk assessment based on available data.

Concluding Statement: metagenomic NGS

- Oral swabs, urine swabs and feces of *M. fuliginosus* bats collected in July 2018 were analyzed using mNGS
- Comparative analysis revealed differences in the virome depending on the sample type
- In oral swabs, bacteriophages were identified as predominant viruses
- Common bat viruses were identified in urine swab and feces samples
- Two CoVs were found in urine swab and feces samples
- One PMV was found in urine swab samples
- The results correspond with typical shedding routes of these viruses

6.3 Evaluation of obtained results in the context of public health

6.3.1 Public Health relevance of conducting virus discovery studies in Sri Lanka

The main goal of this project and associated research on Sri Lankan bats is to assess the prevalence of viruses and their possible impact on public health issues.
According to estimations, only a small percentage of the global virome is even known and the majority of more than 99 % of all viruses is not yet identified [249]. Further research, virus discovery and expansion of viral sequence databases will certainly not be the solution to prevent the future outbreak of EIDs. Nevertheless, the proactive research on yet unknown pathogens may help to identify the causative agents more rapidly in the future and to properly respond to outbreaks.

When exploring databases and tools for conducting the analytical work of this thesis, a research bias became apparent. In terms of geographical distribution, viral data seemed to be mainly available from regions where outbreaks occurred in the past. For the same reason, there also seemed to be a focus on particular virus species and reservoir hosts that are associated with major disease outbreaks. For Sri Lanka, only few molecular biological studies have been conducted on Sri Lankan bats to investigate the presence of viral pathogens.

So far, only viruses or viral sequences of coronaviruses [190] and lyssaviruses [56, 258] have been detected in Sri Lankan flying foxes (*P. medius*), while no studies have been published on other bat species inhabiting Sri Lanka.

With this in mind, the results published in this thesis and the identification of novel pathogens are of high importance as such data also represents the basis for pathogen risk assessment, surveillance and public health measures [250]. Although the results from the investigated bat species did not indicate a prevalence of public health relevant or human pathogenic viruses, preliminary data and evidence of novel virus strains were reported for the first time in Sri Lanka.

6.3.2 Public Health relevance of viral databases

Since the increased occurrence of EIDs in the 20th century the efforts in pathogen and viral research were successively increased and globally available sequence and information databases were established [251]. With the discovery of bats and rodents as important reservoirs for viruses, specific databases such as the bat-associated DBatVir and rodent-associated DRodVir were established [151, 252]. Further important databases and tools are being constantly developed, for instance Nextstrain to track the genomic evolution and spread of viruses including SARS-CoV-2 and MPXV [253], or in the context of Covid-19 databases like LitCovid are being developed to collect current literature [254], the global Covid-19 vaccination dataset [255] and many more. In addition, different international cooperation projects attempt to decipher the global virome and develop a database of viruses in their environmental context [250, 256]. The usefulness of these projects for improving the general public health and outbreak surveillance is being controversially discussed among scientist. For one thing, some doubt the usability of such databases and estimate a low chance of detecting actual EIDs in the massive amounts of data and sequence information. However, one may use the available data from past and recent outbreaks to analyze patterns and to predict upcoming spillover events [257].

All virus sequences obtained in this work were uploaded to the central GenBank database in order to make them generally available and to serve as possible reference for future bat studies in Sri Lanka. With the provided metadata the sequences are also available in the DBatVirus database for bat-associated viruses, filling the gap of yet unreported viruses in bats from Sri Lanka.

6.3.3 Evaluating drivers of viral spillover events

Evaluating the spillover risk of viruses from animals to humans and the risk of EID outbreaks is of crucial importance for public health. Since spillover events are influenced by numerous factors, the investigation of bats as reservoir for potentially human pathogenic viruses is an interdisciplinary research field that includes virology, zoology, ecology and other aspects. A schematic overview of factors enabling the virus spillover from a bat host to humans is visualized in Figure 10.

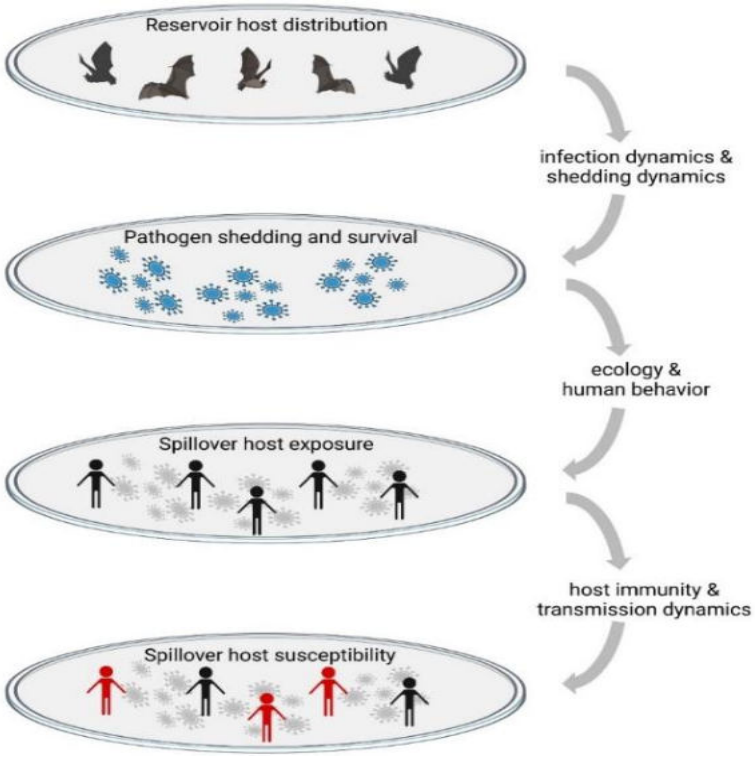

Figure 10: Schematic overview of factors and conditions enabling the transmission of a virus from its natural reservoir to humans. Adapted from [258]. Created with BioRender.com.

As visible in Figure 10, viral spillovers from natural reservoir hosts to humans are complex events depending on multiple factors that need to be considered. First of all, infection dynamics inside the host and shedding dynamics of the viruses are basic and relevant information. For this purpose, not only information and characteristics about the virus should be known, but also zoological aspects and knowledge about the host species should be included. In addition, the survival and persistence of the virus in the environment after shedding is a feature worth investigating. However, successful spillover also requires close contact of humans to the virus, which can be investigated in the context of ecological factors. This includes considering human activities and their intrusion into the host habitat, for instance for agricultural or other land use. After exposure, the susceptibility of humans to the respective virus is important for successful spillover, followed by the potential human-to-human transmission. In this context, a further knowledge of the possible transmission route, the virus structure and possible binding and entry to human cells is worth investigating, as well as the general immunity of humans to the particular virus. As a matter of common knowledge, the number of zoonotic events and related outbreaks has been rising in the past decades and is assumed to further grow in the future [251].

The higher probability of zoonotic spillover events is also a result of numerous, mainly human-made factors such as globalization, loss of biodiversity, destruction of natural habitats and climate change. These changes can increase the general stress levels of bats and alter their behavior, which may both lead to the increased risk of viral shedding and spillover. Understanding the dynamics of zoonoses is therefore a complex field that requires to consider a multitude of associated factors. Therefore, the data collection and compilation in databases needs to be well structured and organized and to provide sufficient relevant background data in order to be beneficial for subsequent research.

The recently developed SpillOver tool aims to rank the risk of spillover based on the variety of factors described before. This tool was used for evaluating partial results of this thesis [203]. The ranking is based on a comparative risk assessment of known zoonotic viruses and yet uncharacterized viruses, considering a number of different risk and impact factors [259]. A prerequisite for the development of such tools and specific databases as described before is the collection not only of single data or sequences but rather the inclusion of numerous relevant metadata. Depending on the respective database and questions, information may be included about the host and host plasticity, relevant geographical and ecological data including climate, seasons and others [259].

6.3.4 Evaluation of sequence analyses conducted in the context of public health

Since no such comparable virome analysis on Sri Lankan bats was available before, the results presented in this thesis can be regarded as important groundwork for virus research and prevalence assessment. Several viral sequence reads of different virus families were obtained by PCR screening, Sanger sequencing, mNGS and with the help of bioinformatic data analysis. Generally speaking, the acquired data can be relevant when conducting a spillover risk assessment for bats inhabiting Wavul Galge cave to humans. The presence of diverse viruses in the bat species was detected in the course of this thesis. This knowledge may raise the awareness of bats as viral reservoir and impel people to generally avoid close contact to the bats or to use proper protective equipment when visiting the cave.

However, the sole presence of viruses does not necessarily imply a public health issue or a possibility of zoonotic events. Such assumptions could lead to the unfounded eradication of

animals (e.g. bats as carrier of CoV including SARS-CoV-2). Instead, further assessments and educational work always need to go along with such new findings.

Therefore, the pathogenic potential of identified viruses was further evaluated based on the available data. Phylogenetic reconstructions were based on either partial sequences or full genes and always included numerous viruses with diverse human pathogenic potential. With these results, an allocation of the novel virus strains within the phylogenetic tree was possible, indicating far distant relationships to highly pathogenic virus species, respectively.

The significance of these results is certainly limited as the obtained sequences from PCR screening and mNGS mostly represented partial sequences of the viral genomes. Sequence analysis and phylogenetic reconstruction of more or less random genomic sequences are less informative compared to the analysis of full genes or genomes. Nevertheless, a rough ranking of the newly identified viruses within a range of known human pathogenic and non-pathogenic viruses was possible. A thorough evaluation of the pathogenic potential requires comprehensive sequence knowledge to identify particular motifs and receptor binding sites. This was realized as part of this thesis by the assembly and characterization of a complete α-CoV genome from NGS data obtained from *M. fuliginosus* bat samples [209]. The in-depth sequence analysis of specific motifs such as the furin cleavage site and ACE2 receptor binding domain in the spike protein gene indicated the inability of the newly detected α-CoV to infect human cells.

In the context of further evaluating the public health relevance, more detailed sequencing data will be necessary to reliably evaluate the human pathogenic potential and risk of spillover from bats of Wavul Galge cave.

Concluding Statement: Evaluation of obtained results

- The established comprehensive sampling strategy can serve as basis for future samplings of bats and other animals in Sri Lanka
- Different novel virus strains were identified for the first time in bats from Sri Lanka
- Preliminary data reveal a rather low human pathogenic potential
- Regular monitoring of bats in Sri Lanka may reveal the presence of public health relevant viruses
- Acquisition of viral sequence data is important as groundwork for future studies

7. Outlook

With the obtained results as presented in this work, the aims of this thesis were accomplished. By conducting three sampling sessions in Wavul Galge, Sri Lanka, bat samples of the sympatric colony were collected for further analysis. Concurrently, the sampling was optimized to establish a comprehensive sampling strategy for future bat studies in this cave (Aim 1). Furthermore, selected samples were analyzed using generic PCR screenings (CoV, PMV) and mNGS (samples from *M. fuliginosus* bats) (Aim 2). Sequencing data was used for further analysis and phylogenetic reconstruction to also evaluate the pathogenic potential (Aim 3). The collected samples as well as the obtained results hold the potential to conduct further studies. A selection of future prospects will be given in the following outlook sections.

7.1 Bat samplings in Wavul Galge using the comprehensive sampling strategy

With regards to future studies on bats in Sri Lanka, the work of this thesis represents an important basis. The three conducted samplings were used to optimize trapping, handling and sampling of bats, to improve sample logistics also regarding sample quality and to increase the safety of the study team working at Wavul Galge cave. The sampling strategy developed from this can be applied in future bat samplings. In general, future studies are envisaged to take place on a frequent basis to cover the influence of seasonal changes and potential shedding patterns and spillover dynamics of particular viruses. Such sampling sessions may be conducted on a monthly basis or quarterly, depending on the planned extent of sampling. Furthermore, and in order to increase the comprehensive knowledge of virus prevalence in the whole of Sri Lanka, other locations should be included in the frequent bat sampling sessions. Therefore, reasonable locations distributed all over the island should be selected and the colony size, composition of different bat species and migrational behavior should be considered.

7.2 Improving the analysis workflow of collected bat samples

Such large-scale studies require the processing and analysis of a high number of samples. As mentioned before, the initial focus of analysis would involve the generic PCR screening of typical bat-associated virus families and mNGS of the samples. With the recent advances in NGS technology and notable increase of sequencing throughput such as with illumina NextSeq, the expenses of mNGS are becoming more and more affordable. Nevertheless, a frequent processing and analysis of all samples collected in future studies would probably exceed the available capacities in many respects. In contrast, the more recent advances in 3rd generation sequencing and especially the nanopore-based MinION sequencing have reduced the costs and expenditure of time for sequencing and data analysis. The main drawback of low sequencing accuracy is targeted by constantly improving the nanopore system and associated currency measurement of the nucleotides. This may be compensated for by using the amplification sequencing approach (AmpliSeq). With an upstream amplification of viruses suspected in the samples, followed by sequencing of the amplified products, this method can be used to efficiently screen simultaneously for a number of viruses of interest. This described procedure of targeted sequencing can replace the application of single PCRs for each virus or virus family on the one hand and facilitate the sequencing of viruses on the other hand by analyzing "targeted viromes" [168].

In the context of future bat studies, one aim is to develop an AmpliSeq Bat Panel for prevalent viruses in bats from Sri Lanka and to subsequently screen the samples collected frequently from the different sampling sites. The application of more laborious and expensive mNGS would be necessary only when establishing bat research on a novel sampling site or for a novel bat species, in order to initially discover the complete virome and to include the prevalent viruses in the AmpliSeq Bat Panel. Therefore, the mNGS data already available, including the results of this thesis, will serve as important basis for designing and establishing this panel. In the following process, particular samples may also be sequenced with mNGS if a result from the AmpliSeq approach reveals relevant data that require further in-depth sequencing or whole genome sequencing. In any case, the general workload can be reduced by the inclusion of this method. With the general inclusion of all viruses detected so far in Sri Lankan bats in one

collective panel, such screening once again holds the potential to reveal important information about the host range of viruses, their seasonality or geographical distribution in Sri Lanka.

The following Figure 11 illustrates the planned procedure for future bat samplings in Wavul Galge cave or other sampling locations in Sri Lanka and also demonstrates the workflow of subsequent sample analysis.

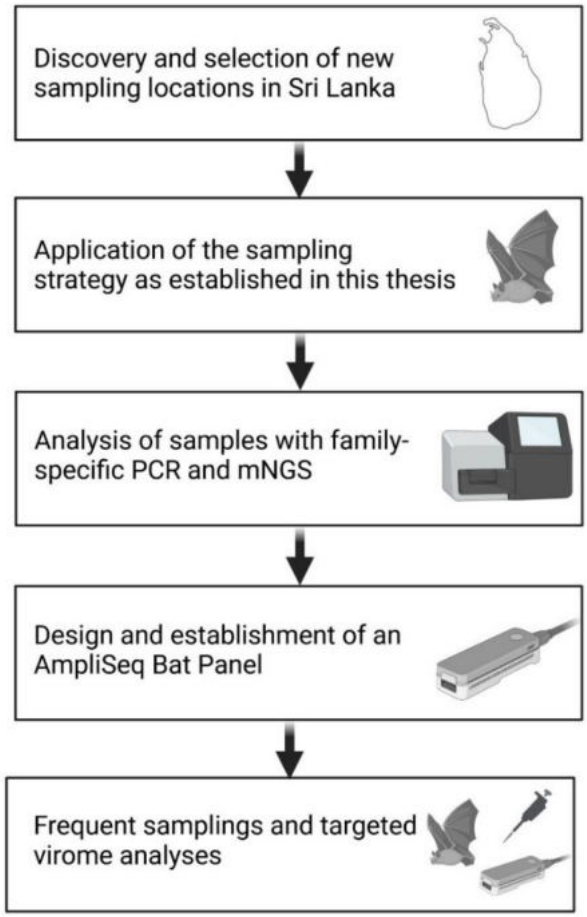

Figure 11: Schematic procedure of the future bat samplings in Wavul Galge cave and other locations and subsequent sample processing and analysis. Created with BioRender.com

7.3 Further virome analyses of bat samples from Wavul Galge

In the course of this thesis, the virome of *M. fuliginosus* bats from Wavul Galge cave was analyzed. This bat species is of particular interest as it is globally distributed and known to harbor numerous viruses including public health relevant viruses.

However, Wavul Galge cave represents a sympatric colony of several bat species of the genera *Miniopterus*, *Hipposideros*, *Rousettus* and *Rhinolophus*, living in close contact to each other. In the context of virological and ecological research questions, it may be of interest to also analyze the viromes of the other bat species inhabiting this cave and to compare the respective virome composition between the bat species. Based on the results already available, the identified viruses seemed to be prevalent in only one bat species of the sympatric colony. Comparing the complex virome data of each species will help to prove or refute this observation. In terms of virology, it will be of interest to analyze the host range of the respective virus species. With regard to ecology, analyzing the dynamics between the different bat species inhabiting Wavul Galge will be of interest to understand how closely they interact and if the presumed viral exposure from one bat species to another is even possible. Against this background different sample types are available from all bat species, allowing to investigate different shedding patterns not only to humans but also between the bat species in the cave.

7.4 Further ecological and virological research prospects

Comparing the microbiome and nutrition of the different bat species may reveal differences and allow for insights into their diet, which in turn allows for conclusions regarding their contribution to ecosystem services. The comparative metagenomics between different time points can reveal characteristic shedding patterns of particular viruses throughout the year. This may be correlated with different ecological factors and the reproductive cycles of the bats, but can also be a result of external stressors, human intrusion into natural bat habitats and climate change which can all increase the possibility of viral shedding [83, 260].

In previous samplings, also ectoparasites from each bat species were collected for further analyses. As an outlook for future studies, these parasites may be analyzed with similar workflows in order to determine the prevalence of viruses. Further ecological investigations

could determine the respective parasite species and reveal if they are limited to a specific bat species and if they carry the same viruses.

7.5 Applying further virus detection methods on collected bat samples

Different further virus detection methods could be applied on the collected bat samples to obtain a more comprehensive analysis and understanding of viral prevalence. The application of different methods enables a comprehensive characterization of newly detected viruses. Each approach entails advantages and drawbacks in the analysis and result outcome, while the combination of different methods can complement the informative values of the analysis. The methodical focus of this thesis was explicitly on the application of molecular biological methods and bioinformatic analysis of sequence data. This included the more targeted screening of samples with family-specific PCRs and the mNGS as open view approach. In the future, other virus detection methods such as cell culture, electron microscopy and serology should be envisaged to complement the analysis. These rather elaborate methods can be conducted with only those samples that were previously tested positive for particular viruses in PCR or mNGS analyses. The propagation of a virus in cell culture can provide a virus isolate as the basis for further analyses (e.g. titration, full genome sequencing). Concurrently, the analysis of samples with electron microscopy as imaging technique can demonstrate the presence of actual viral particles. Also, serological analyses of collected serum samples can reveal additional information about the serotypes of the viruses.

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9. List of Publications

Detection of Alpha- and Betacoronaviruses in* Miniopterus fuliginosus *and* Rousettus leschenaultii*, two species of Sri Lankan Bats

Therese Muzeniek, Thejanee Perera, Sahan Siriwardana, Dilara Bas, Fatimanur Kaplan, Mizgin Öruc, Beate Becker-Ziaja, Franziska Schwarz, Gayani Premawansa, Sunil Premawansa, Inoka Perera, Wipula Yapa, Andreas Nitsche and Claudia Kohl.

Vaccines 2021, 9, 650. https://doi.org/10.3390/vaccines9060650

Full Genome of batCoV/MinFul/2018/SriLanka, a Novel Alpha-Coronavirus Detected in* Miniopterus fuliginosus*, Sri Lanka

Therese Muzeniek, Thejanee Perera, Sahan Siriwardana, Dilara Bas, Fatimanur Kaplan, Mizgin Öruc, Beate Becker-Ziaja, Inoka Perera, Jagathpriya Weerasena, Shiroma Handunnetti, Franziska Schwarz, Gayani Premawansa, Sunil Premawansa, Wipula Yapa, Andreas Nitsche and Claudia Kohl.

Viruses 2022, 14, 337. https://doi.org/10.3390/v14020337

Paramyxovirus Diversity within One Population of* Miniopterus fuliginosus *Bats in Sri Lanka

Therese Muzeniek*, Thejanee Perera*, Sahan Siriwardana, Fatimanur Kaplan, Dilara Bas, Mizgin Öruc, Beate Becker-Ziaja, Inoka Perera, Jagathpriya Weerasena, Shiroma Handunnetti, Franziska Schwarz, Gayani Premawansa, Sunil Premawansa, Wipula Yapa, Andreas Nitsche and Claudia Kohl
* equal contribution

Pathogens 2022, 11, 434. https://doi.org/10.3390/pathogens11040434

First Complete Cytochrome B Sequences and Molecular Taxonomy of Bat Species from Sri Lanka

Thejanee Perera, Franziska Schwarz, Therese Muzeniek, Sahan Siriwardana, Beate Becker-Ziaja, Inoka C. Perera, Shiroma Handunnetti, Jagathpriya Weerasena, Gayani Premawansa, Sunil Premawansa, Andreas Nitsche, Wipula Yapa and Claudia Kohl

Animals 2022, 12, 1674. https://doi.org/10.3390/ani12131674

Comparative virome analysis of individual shedding routes of* Miniopterus fuliginosus *bats inhabiting the Wavul Galge Cave, Sri Lanka

Therese Muzeniek, Thejanee Perera, Sahan Siriwardana, Dilara Bas, Fatimanur Bayram, Mizgin Öruc, Beate Becker-Ziaja, Inoka Perera, Jagathpriya Weerasena, Shiroma Handunnetti, Franziska Schwarz, Gayani Premawansa, Sunil Premawansa, Wipula Yapa, Andreas Nitsche and Claudia Kohl

BioRxiv, 2022. https://doi.org/10.1101/2022.09.21.508883

One Health Approach for the sampling of different bat species living in a sympatric colony

Thejanee Perera*, Sahan Siriwardana*, Therese Muzeniek*, Beate Becker-Ziaja, Dilara Bas, Fatimanur Bayram, Mizgin Öruc, Inoka Perera, Jagathpriya Weerasena, Shiroma Handunnetti, Franziska Schwarz, Gayani Premawansa, Sunil Premawansa, Wipula Yapa, Andreas Nitsche and Claudia Kohl

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BioRxiv, 2022. https://doi.org/10.1101/2022.09.22.508887

10. Figures

11. Tables

12. Annex

12.1 Publication I

One Health Approach for the sampling of different bat species living in a sympatric colony

12.2 Publication II

Paramyxovirus Diversity within One Population of **Miniopterus fuliginosus** ***Bats in Sri Lanka***

12.3 Publication III

Detection of Alpha- and Betacoronaviruses in **Miniopterus fuliginosus** ***and*** **Rousettus leschenaultii,** ***two species of Sri Lankan Bats***

12.4 Publication IV

Full Genome of batCoV/MinFul/2018/SriLanka, a Novel Alpha-Coronavirus Detected in **Miniopterus fuliginosus,** ***Sri Lanka***

12.5 Publication V

Comparative virome analysis of individual shedding routes of **Miniopterus fuliginosus** ***bats inhabiting the Wavul Galge Cave, Sri Lanka***

One Health Approach for the sampling of different bat species living in a sympatric colony

Thejanee Perera [1+], Sahan Siriwardana [2+], Therese Muzeniek [3+], Beate Becker-Ziaja [4], Dilara Bas [3], Fatimanur Bayram [3], Mizgin Öruc [3], Inoka Perera [2], Jagathpriya Weerasena [1], Shiroma Handunnetti [1], Franziska Schwarz [3], Gayani Premawansa [5], Sunil Premawansa [2], Wipula Yapa [2], Andreas Nitsche [3] and Claudia Kohl [3*]

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Keywords: bat sampling, bat colony, virology, zoology, ecology

Abstract

Bats are important contributors to the global ecosystems; at the same time, they are known to be a natural reservoir host for a number of human pathogenic viruses. These and many other unique features make them an interdisciplinary research object in the context of One Health, comprising zoology, ecology, virology, microbiology, molecular biology, immunology and public health issues. Performing field studies for bat research often aims to cover several of these topics and requires the combination of specific expertise in different fields. We carried out three individual field studies in Wavul Galge cave (Koslanda, Sri Lanka), where several bat species roost sympatrically. The main goals were to study the bat colony for ecological aspects and to sample bats for virological and molecular biological analyses. In the course of the field studies, we optimized the sampling procedure regarding safety aspects, a preferably low impact on the captured bats and an improved output of high-quality samples for further analysis. Different sampling methods and procedures were compared in order to establish a suitable strategy for frequent sampling and monitoring of these bats. In the present case study, we report on this process of optimizing our field work and provide suggestions for bat sampling methods that cause comparably less stress for the captured animals. We also report on constraints and obstacles encountered during the practical implementation and possible measures to overcome these.

With these practical experiences, we hope to give support to other interdisciplinary research teams preparing for bat field work. Furthermore, we emphasize the need for the respectful treatment of the animals and minimized disturbance of their natural habitat when carrying out sustainable bat research.

Introduction

Bats of the order *Chiroptera* are mammals with the second-highest species diversity after rodents and are globally distributed [1]. Unique features like their ability to fly, migratory habits and longevity make them a promising topic in zoological and ecological research [2]. In addition, bats are known to be the natural reservoir hosts of numerous pathogens including zoonotic viruses causing diseases in humans [3].

External factors like globalization, climate change, loss of biodiversity and exploitation of the bat habitats by urbanization increase contact between humans and bats, and there is an ongoing

61 debate about whether these factors increase the risk of spillover of pathogens to humans [4, 5].
62 A long-term co-evolution of bats and viruses is assumed, resulting in an adapted immune
63 system and low susceptibility of bats to infection with these pathogens and to the development
64 of symptomatic diseases [6]. This ability of the bat immune system to control the persisting
65 viruses without developing diseases is an interesting research field in terms of immunology [7].
66 As a result, research on bats in general is an interdisciplinary field that may combine different
67 research questions in One Health, e.g. regarding ecology, zoology and environmental sciences
68 with virology, microbiology, molecular biology, immunology and public health-related issues.

69 Bats are social animals and live in colonies in different roosting sites including caves. Caves
70 are known to offer largest known mammalian congregations, sometimes exceeding hundreds
71 of thousands of bats roosting in a single location. In these dwellings, bats of different species
72 often roost sympatrically. Although these different species usually occupy different locations
73 inside a cave roost and are essentially separated, they have closer contact during inflight and
74 outflight and also share aerosols. This may facilitate inter-species transmission of viruses and
75 increase the general prevalence and persistence of viruses in these mammals [8]. The interaction
76 between ecological factors and virological investigations is therefore an important part of the
77 interdisciplinary research.

78 With more than 30 species, bats represent one third of the mammalian species inhabiting the
79 island of Sri Lanka [9]. They contribute to the agro-ecosystems by pollination, seed dispersal
80 and insect control, which renders them particularly interesting for ecological research and
81 conservation biology [9, 10]. Although the general ecology of bats in Sri Lanka is well studied,
82 so far only few scientific studies on their role as viral reservoirs have been published [9, 11–
83 14]. With this case study, we present the establishment of an interdisciplinary field study on bat
84 species inhabiting Wavul Galge cave (Koslanda, Sri Lanka). In three individual sampling
85 sessions in 2018 and 2019, catching and sampling of bats were optimized in terms of ecological,
86 virological and molecular biological research aspects, personal safety of the research team and
87 reduction of harm and stress for the bats during the sampling process. The planning and
88 conducting of the sampling sessions were based on specific knowledge by experienced
89 zoologists regarding bat behavior, capturing and handling, in combination with the
90 requirements of high-quality sampling for molecular biological and virological analyses. A few
91 guidelines for the catching and handling of bats are available, providing the groundwork for the
92 preparation of sampling sessions [15–17]. However, the field study conditions strongly depend
93 on the environmental circumstances and the respective scientific question that has to be

answered. The number of captured bats and the extent of different samples taken per bat may differ. This case study describes the experience we gained throughout this process of optimizing our sampling sessions on bat research. The methods and insights that we describe here may help other research teams when engaging in bat field work also in other countries and when preparing and deciding on the different available sampling strategies.

Methods

The sampling of bats was carried out in Wavul Galge (Koslanda, Sri Lanka, 6°41'50.2"N 81°03'51.0"E), an underground cave in the interior of Sri Lanka (Figure 1) where bats of the genera *Miniopterus*, *Hipposideros*, *Rhinolophus* and *Rousettus* roost sympatrically.

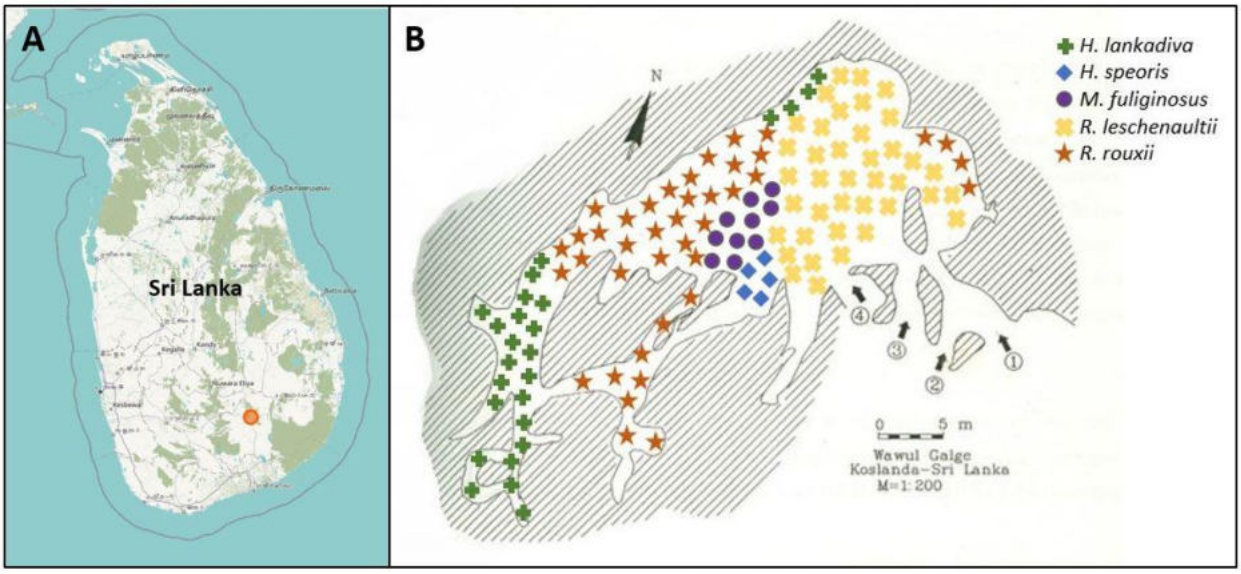

Figure 1: A: Location of Wavul Galge cave (marked in red) on the island of Sri Lanka (© OpenStreetMap in accordance with the Open Data Commons Open Database License). B: Schematic plan of the underground cave Wavul Galge, Sri Lanka. The roosting locations of the different bat species in the cave and the four entrances to the cave (1–4) are shown in the map.

Sampling permission was issued by the Department of Wildlife Conservation, Sri Lanka (permit No. WL/3/2/05/18, issued on 10 January 2018) and the sampling was conducted in accordance with the guidelines and regulations of the Fauna and Flora Protection Ordinance, Sri Lanka.

A total of three sessions for sampling bats were performed in March 2018 (03/18), July 2018 (07/18) and January 2019 (01/19) in order to cover different seasons of the year. During these sampling sessions, we optimized our sampling strategy stepwise and compared different

119 methods and materials. Cost–benefit considerations were made based on stress exposure to the
120 bats, safety precautions for the research team and practicability.
121 An overview of equipment and material used for conducting the bat samplings is given in the
122 supplementary material.
123
124 ***Personal protective measures***
125 Adequate personal protective equipment (PPE) was used at all times during the catching and
126 sampling of the bats, both for the protection of the sampling team (to prevent zoonoses) but
127 also to protect the bats (risk of anthropozoonoses).
128 During the bat catching procedure, head coverings (baseball cap, hats or comparable) and FFP3
129 masks were worn as protection from bat droppings and aerosols. Solid work gloves were used
130 to transfer the bats from the nets to the bat holding bags.
131 During the sampling procedure of the bats, different gloves were worn to allow for a more
132 flexible handling of the bats. Instead of rigid work gloves, a layer of conventional lab gloves
133 followed by polyurethane-coated protection gloves were worn. Another layer of lab gloves was
134 used on top to be easily cleaned or changed in the case of contamination.
135
136 ***Catching procedure***
137 For the catching of bats, either home-made hand nets or a harp trap (Bat Conservation and
138 Management Inc., Carlisle, Pennsylvania, USA) were used and their feasibilities were
139 compared. Furthermore, we compared catching and sampling of bats either in evening sessions
140 at dusk when they left the cave or in morning sessions at dawn when bats returned to the cave.
141 Captured bats were kept in holding bags until further processing; for this a sampling tent was
142 set up near the cave entry. Bats were always released directly in front of the cave after sampling
143 was finished. The catching procedure is visualized in Figure 2.
144

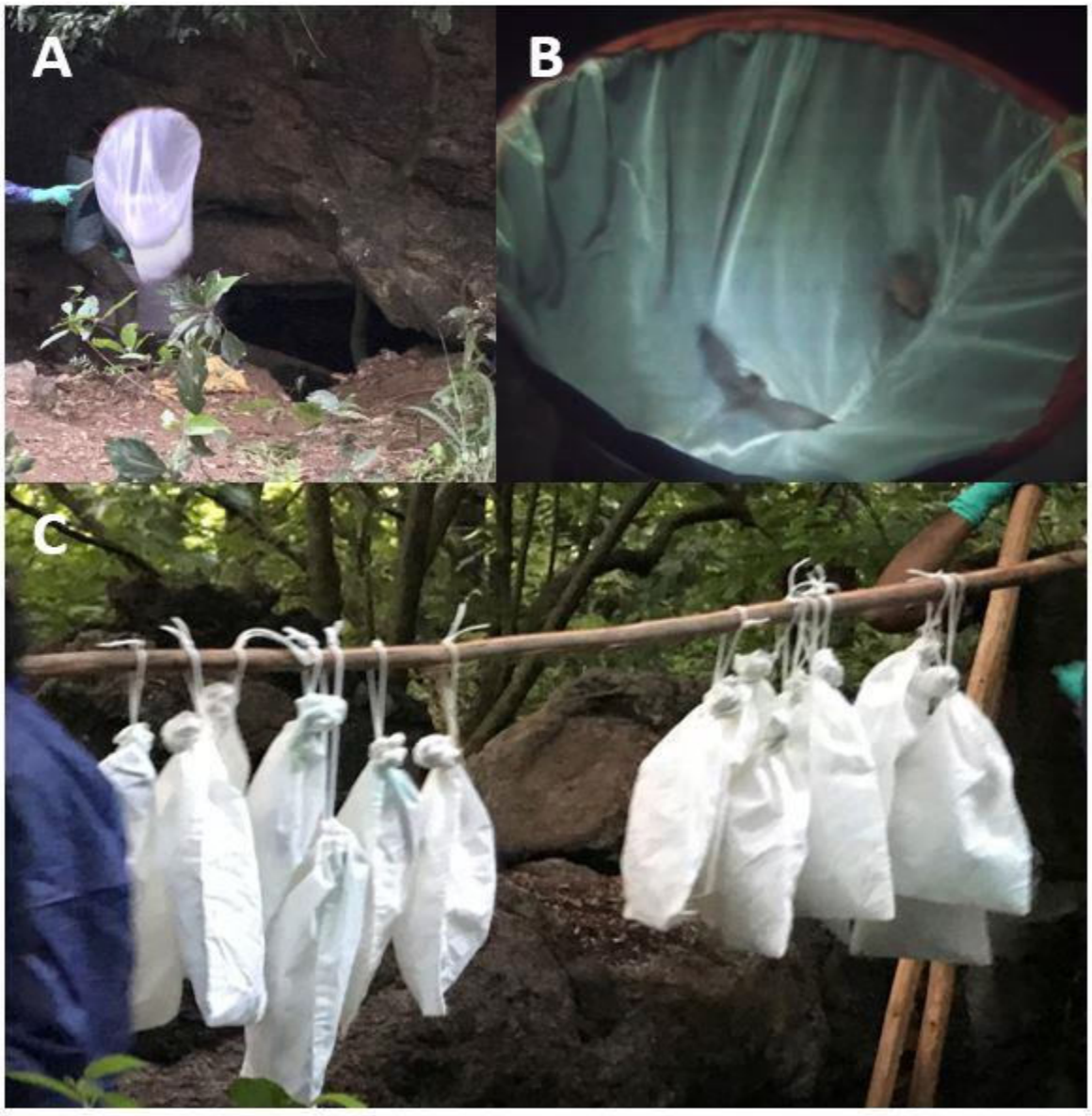

Figure 2: Steps of the bat catching procedure. A: Catching of bats in front of the underground cave Wavul Galge using a hand net. B: Two bats trapped in a hand net. C: Transport of bats in holding bags to the tent for taking samples. © Beate Becker-Ziaja, Therese Muzeniek

Sampling of bats and documentation

To every individual bat captured an internal number was assigned and basic parameters were assessed and documented as listed in Table 1.

159

160 Table 1: Parameters recorded during the sampling of bats from Wavul Galge cave, Sri Lanka.

Documented parameter	Selection (if applicable)
Key data	Sampling date, time, internal bat number
Macroscopic species determination	*Miniopterus phillipsi, Rhinolophus rouxii, Hipposideros speoris, Hipposideros lankadiva, Rousettus leschenaultii*
Collected sample types	Oral swabs, rectal swabs, urine swabs, feces, blood, ectoparasites
Sex	Male, female
Age class	Juvenile, sub-adult, adult
Health condition	Good, fair, poor
Morphometric measurements	Weight (g), forearm length (cm from elbow to wrist)
Additional notes	e.g. Injuries, pregnant, lactating

161

162 According to the issued sampling permission, oral swabs, rectal swabs, urine swabs, fecal
163 pellets, blood samples and ectoparasites were collected from the animals if possible.
164 For swabbing, we compared sterile Minitip FLOQSwabs® (Copan Diagnostics, Murrieta, CA,
165 USA) with CleanFoam® swabs (ITW Texwipe, Kernersville, NC, USA) which were not sterile
166 but had to be autoclaved in advance. FLOQSwabs® were available in one size and were used
167 during the 03/18 sampling session for taking all kinds of swabs from the bats. CleanFoam®
168 swabs of different sizes and shapes were used during the 07/18 and 01/19 sampling sessions.
169 For taking oral swabs and urine swabs, CleanFoam® round swabs with a diameter of 3.8 mm
170 were used. Rectal swabs were taken by using CleanFoam® spear-shaped swabs with a
171 maximum diameter of 2.5 mm (for more details see supplementary material).
172 Suitable swabs were selected based on their handiness during sampling and also with regard to
173 the subsequent laboratory processing of the samples. The shapes of the three described swab
174 types are compared in Figure 3.
175

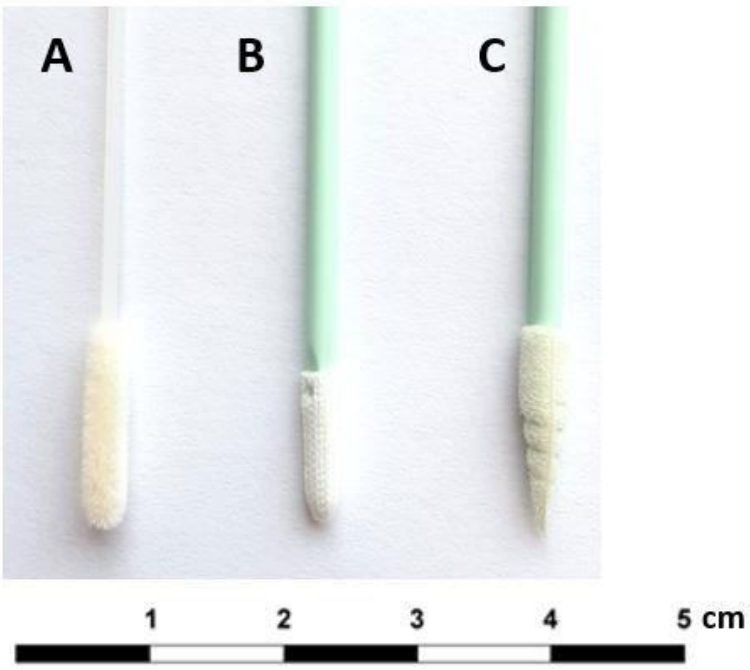

Figure 3: Comparison of different swab types used during the bat sampling sessions. A: FLOQSwabs® used during the 03/18 bat sampling session for all sample types. B: CleanFoam® round swab used during the 07/18 and 01/19 bat sampling sessions for taking oral swabs and urine swabs. C: CleanFoam® spear-shaped swab used during the 07/18 and 01/19 bat sampling sessions for taking rectal swabs.

Fecal pellets were collected with forceps from the bat holding bags if available. Forceps were cleaned afterwards and wiped using ethanol. Ectoparasites were also collected with forceps directly from the bat fur if possible. For taking blood samples, small sterile cannulas (Sterican 30G, 0.3x12mm, B. Braun, Melsungen, Germany) were used. In the first two sampling sessions 03/18 and 07/18, conventional glass capillaries were used for sample collection, stored in 2 mL screw cap microtubes (Sarstedt Inc., Nümbrecht, Germany) and frozen for transportation. In the 01/19 sampling session, blood was collected on Whatman® protein saver cards, stored and transported in plastic bags together with silica gel bags at room temperature.

Examples of the bat sampling procedure are given in Figure 4.

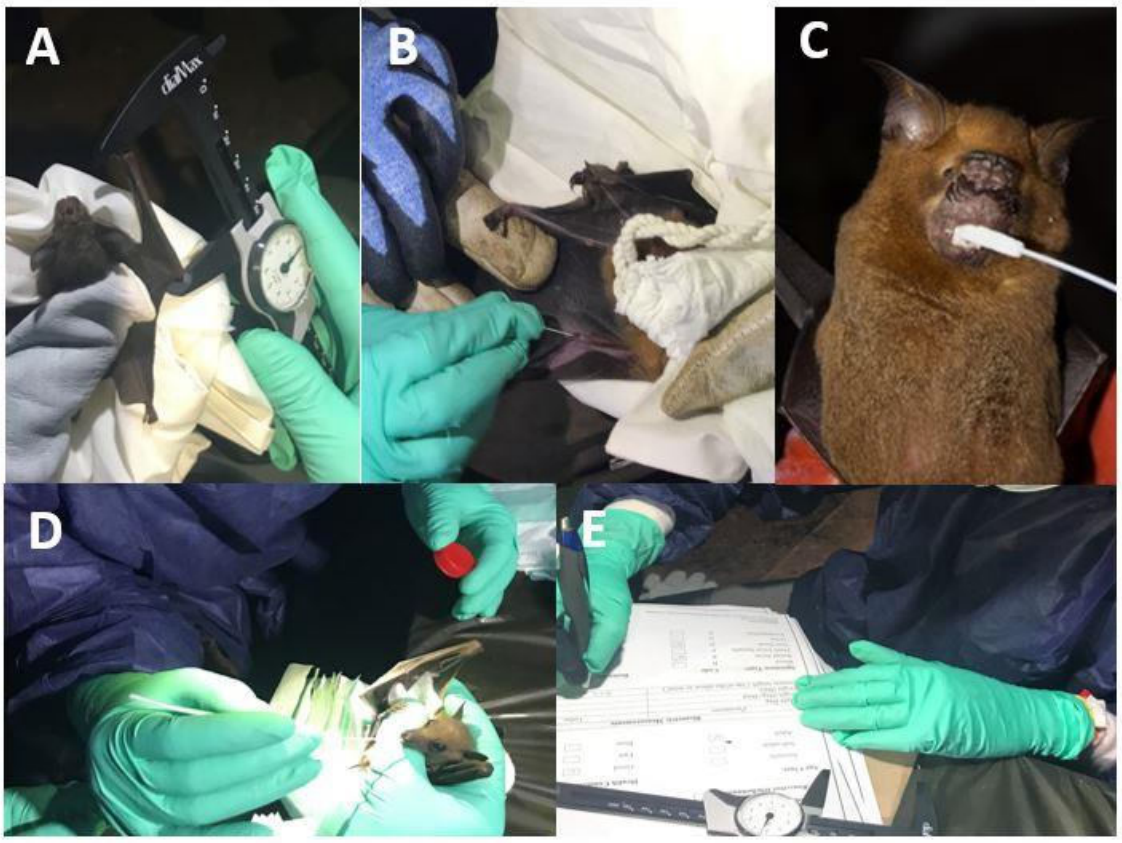

Figure 4: Examples of the bat sampling procedure. A: Taking forearm length measurements of an *R. rouxii* bat. B: Drawing blood from an *H. speoris* bat by venipuncture. C: Oral swabbing of an *H. speoris* bat. D: Oral swabbing of an *R. leschenaultii* bat. E: Documenting the taken measurements on the record sheet. © Beate Becker-Ziaja, Sahan Siriwardana

All collected sample types described above were stored in 2 mL screw cap microtubes and stored without any additives for transportation. During the two first sampling sessions in 03/18 and 07/18, Va-Q-Tcon cooling boxes (va-Q-tec, Würzburg, Germany) with -80°C cooling packs were used for sample storage and transport. In the 01/19 sampling sessions, a dry shipper VOYAGEUR Plus (Cryopal, Bussy Saint Georges, France) with absorbed liquid nitrogen was used for storage and transportation. For this purpose, samples were stored in a nylon stocking and collected samples were separated with a knot to differentiate between the samples per bat. Filled nylon socks were put into the dry shipper for storage. The sample storage is shown in Figure 5.

Figure 5: Storing of collected bat samples in a dry shipper by using nylon socks. © Therese Muzeniek

The collected samples were subsequently handled in laboratories of the biosafety level 2 and processed with regards to research questions from zoology, ecology, virology, microbiology, molecular biology, immunology and public health-related issues.

Results

In a total of three sampling sessions, different bat species from Wavul Galge cave, Sri Lanka, were sampled, while the catching and sampling strategy was optimized stepwise. Table 2 gives an overview of the three sampling sessions including basic conditions and total number of sampled bats. Table 3 gives a more detailed overview of samples collected per bat species.

Table 2: Overview of the conducted bat sampling sessions at Wavul Galge cave, Sri Lanka.

Sampling session	03/2018	07/2018	01/2019
Sampling days (total)	2 days	4 days	5 days
Sampling time	Dusk	Dusk	Dusk/dawn
Sampled bats (total)	82	200	107

Table 3: Overview of the three conducted sampling sessions, including number of collected samples per bat species.

		Oral swabs	Rectal swabs	Feces	Urine swabs	Blood	Ectoparasites
***Hipposideros* spp.**	**Total (bats)**						
03/2018	**3**	3	3	0	2	0	0
07/2018	**1**	1	0	0	0	0	0
01/2019	**22**	22	16	7	6	21	3
M. phillipsi	**Total (bats)**						
03/2018	**3**	3	3	0	0	0	2
07/2018	**188**	188	116	77	102	10	10
01/2019	**31**	31	4	27	11	23	10
R. leschenaultii	**Total (bats)**						
03/2018	**9**	9	9	2	2	1	5
07/2018	**11**	11	11	0	2	3	5
01/2019	**20**	20	16	3	6	18	10
R. rouxii	**Total (bats)**						
03/2018	**67**	67	60	8	6	0	10
07/2018	**0**	0	0	0	0	0	0
01/2019	**34**	34	16	17	6	23	10

As shown in the overview in Table 2, the total days of bat sampling were extended with each sampling session. In doing so, our main goal was not to increase the number of sampled bats; on the contrary, we even reduced the total number of bats during the last visit (Table 3). Instead, we focused on extended sampling including blood samples and collecting feces pellets and ectoparasites, which took considerably more time compared to the other samplings.

In addition, we aimed to balance the number of captured bats per species, which was optimized in the last sampling session in 01/2019. While the use of a harp trap for bat sampling was tested once during the first sampling session, capturing of bats was generally realized using hand nets at the entrances of the cave. This catching procedure also facilitated to control the number of captured individuals per bat species.

Sampling procedure and documentation

For thorough documentation of the bat samplings, we included different parameters as specified in the methods section (Table 1). These parameters were mainly recorded to cover the ecological and epidemiological aspects of the field study and to obtain an overview on the age, sex and species distribution at different sampling time points. Furthermore, if viruses were

identified in subsequent molecular analyses, an epidemiological correlation of the recorded attributes to the positive findings was attempted.

The most suitable sampling material was selected based on different criteria. For swabs the main factors were sterility, absorbing capacity, size and shape. The tested CleanFoam® swabs (round shape) appeared to be most suitable for taking oral swab samples, while the CleanFoam® swabs (mini spear shape) facilitated taking rectal swabs also from smaller bat species like *M. phillipsi*.

For the taking of blood samples, we selected a needle diameter applicable on the smallest bat species which was also suitable for taking blood from larger bats. Although blood collection on filter paper was rather unwieldy compared to using the capillary system, it revealed substantial advantages regarding sample storage, transportation and subsequent sample preparation for lab analyses. Therefore, this method was selected to be the preferred and most suitable one for taking blood.

Discussion

Field studies for zoological and molecular biological bat research

With the field studies described, we aimed to capture and sample bats inhabiting Wavul Galge cave in the interior of Sri Lanka. Our goal was to optimize the sampling procedures covering interdisciplinary One Health questions from the fields of zoology, ecology, virology, microbiology, molecular biology, and immunology while minimizing the disturbance to the natural habitat of the bat populations. Sampling was also optimized to increase the safety of the research team. For this purpose, suitable PPE was available. Also, prophylactic measures were considered, including full rabies vaccination and knowledge of hospitals located nearby in case of emergencies. In order to protect the bats from anthropozoonoses, the research team was allowed to work at the cave only in good health condition.

At the same time, we optimized the processes of capturing bats and their sampling in a way to obtain optimal sample quality with minimized stress for the animals and maximum safety of the research team.

Optimizing the catching of bats

In general, we observed that a smaller number of bats captured per day was beneficial as it reduced the individuals' total time spent in the holding bags. In return, the number of total days per sampling session was increased. With the low sampling throughput we optimized for our future sampling sessions, the use of hand nets for bat catching appeared to be sufficient.

Advanced sampling equipment would be recommended if a higher number of bats was desired, while a harp trap may be generally advantageous over mist nets regarding the risk of injuring bats that are trapped inside the nets. Mist nets should not be used closer to the entrances of the cave when there is peak exodus of bats, as this may result in entanglement of a large number of bats in the net while releasing them within a short period of time is not feasible.

When planning the duration of the sampling session and the maximum number of bats sampled per day, other parameters like the requested sample types (swabs, blood samples or ectoparasites), the size of the research team and the general sampling conditions (morning, evening and season) have to be considered as they all influence the processing time per bat. Depending on the samples taken, the processing of one bat usually took between five and ten minutes.

During the first two sampling sessions, only evening sessions were performed as the setup of the sampling station and preparation for the catching was facilitated during daytime. The bat catching itself was easy to coordinate, as the different bat species leave the cave in a more or less specific order. Also, sampling itself was more convenient to endure for hours, as temperatures and humidity decrease during the night.

With the last sampling session in 01/2019, we set up sampling in the early morning instead of the evening. Despite the above-mentioned advantages of evening sessions, we observed a number of advantages of morning sessions. The main advantages were observed in the bats as they returned to the cave in the morning, satiated after feeding and calmer compared to the evening. As a result, it was easier to keep the bats in the holding bags until processing and the handling seemed to be less stressful for the bats. When catching bats in the morning, they were more likely to defecate into their holding bags. Thus, the number of feces samples was increased and fewer rectal swab samples had to be taken. Drawing blood especially from small bat species like *Miniopterus phillipsi* was notably easier in the morning sessions using daylight than in evening sessions when only artificial light and head torches were available. The dependence on artificial light was a general disadvantage of the evening sessions, as it attracted numerous insects and at times other wild animals like elephants passing nearby, forcing us to move the bat sampling tent several times to other locations. Therefore, sampling in morning sessions appeared to be safer for the research team, also with regard to personal protection. Due to the rising temperatures over the course of the day, the bat processing was terminated around 10 am to prevent the bats from remaining captured in holding bags too long during the heat of the day. When releasing the bats after processing in the morning, special attention had to be taken to release the bats inside the cave in order to facilitate their orientation and to ensure bats fly

315 directly into the cave. If bats do not enter the cave and circle around the cave in the morning,
316 they could be easy prey for avian predators.

317 Since it was aimed at carrying out bat research focusing on One Health, comprising ecological
318 aspects and virological questions concurrently, different approaches had to be considered for
319 the field studies. A general overview of the bat species inhabiting the cave including some
320 ecological characteristics that were discovered during these and previous sampling sessions is
321 given in Table 4.
322

323 Table 4: Overview of some ecological characteristics of the five bat species inhabiting Wavul
324 Galge cave, Sri Lanka, as observed or confirmed during the three bat sampling sessions

	Hipposideros lankadiva	***Hipposideros speoris***	***Miniopterus phillipsi***	***Rousettus leschenaultii***	***Rhinolophus rouxii***
Nutrition	Insectivorous	Insectivorous	Insectivorous	Frugivorous	Insectivorous
Outflight order	2nd	2nd	1st	3rd	1st
Reproduction cycles	March – May	September – October	July – August	April, September	September – October
Migration	No	No	Yes	No	Yes

325

326 For virus discovery in the bat samples and comparing the virus prevalences among each other,
327 a representative number of individuals per bat species seemed to be useful. However, the
328 proportions of the bat species are not evenly distributed and fluctuate significantly over the
329 year, which is of interest to the ecological research on this sympatric bat colony. For example,
330 during the sampling session in 07/2018 we recognized a peak in *M. phillipsi* bats inhabiting the
331 cave. As was discovered earlier, this bat species uses Wavul Galge as pre-maternity cave during
332 this time of the year (compare Table 4).
333 Field studies during this time can be of interest under ecological aspects but as well for
334 virological questions, as a number of bats from different surrounding satellite colonies gather
335 and mix in this cave. It is interesting to investigate the virus prevalence and shedding pattern at
336 this time compared to other periods of the year. However, it should be considered that a high
337 number of these bats are pregnant at that time point and therefore should not be stressed, for
338 instance by taking blood.
339 These examples show that zoological aspects and molecular biological questions have to be
340 balanced thoroughly when preparing the respective field studies. The expertise and experience
341 of zoologists and ecologists are important contributions to virological research in the field. In
342 the following, we discuss the molecular biological requirements that influenced the bat
343 sampling process.
344

Bat sampling for subsequent molecular biological and virological analyses

With the samples taken from bats, it was intended to perform different molecular biological and virological investigations including PCR, metagenomic NGS, virus isolation in cell culture and serological analyses. For this spectrum of analyses, we aimed to take different sample types in optimal quality. For successful virus isolation, intact virus particles are required. Furthermore, intact nucleic acids with low degradation are needed for PCR and NGS analyses. Both the virus particles and nucleic acids are susceptible to high temperatures and may degrade quickly. A continuous cold chain was therefore essential for the sample quality and successful laboratory analysis, especially in a tropical country like Sri Lanka. We observed that even professional cooling bags could not maintain an adequate temperature for a longer period of time. A suitable solution was therefore the use of a dry shipper that maintained the temperature longer. This shipper contained material adsorbing liquid nitrogen, and the cooling was achieved by its gradual evaporation into the storage area of the shipper. By this, an adequate cooling temperature could be maintained for several weeks. In addition, in accordance with current IATA regulations, appropriate dry shippers are suitable for transport of the samples via airplane, facilitating the sample logistics.

Due to the limited size of the available dry shipper, its limited capacity for sample storage and transportation had to be considered when planning the respective field trips. Therefore, a beneficial solution for taking blood samples was to collect drops on protein saver cards. These were stored in a single plastic bag per card and transported at room temperature. It was important to avoid humidification of the dried blood; for this we used desiccants like silica gel sachets in each bag.

Other samples such as oral swabs, rectal swabs, urine and feces were snap-frozen within the dry shipper without any additives. Liquid was added later during the preparation process of the samples in the laboratory. In terms of molecular biological analyses, this method did not impair the outcome of the analyses as we were able to detect different viruses via PCR and in NGS analyses [11, 12, 18]. The presence of intact virus particles has not been tested yet, and virus isolation in cell culture will be performed in future analyses. However, samples may also be stored in suitable viral transport medium or PBS in order to maintain virus integrity. Care must be taken to use a medium suitable for the variety of subsequent analyses.

It is a common technique to take wing punches of the sampled bats for species identification purposes [19]. However, it was discovered that oral swabs were also suitable for the analysis based on cytochrome b PCR and sequencing [20]. By this, it was possible to reduce invasive bat sampling to a minimum.

Concerning different kinds of tested swabs, we observed that a less absorbing material was beneficial especially for the subsequent sample preparation. For the tested FLOQSwabs®, additional 300 µL of liquid had to be added in order to retain sufficient volume for Nucleic Acid extraction. In contrast, the preferred CleanFoam® swabs absorbed the liquid samples (urine, saliva etc.) but clearly less of the liquid in the subsequent processing. In addition, the stable handle and special forms of the different CleanFoam® swabs facilitated sample taking, especially for small bat species. However, other swab brands may be suitable depending on the respective sampling setup. It is important to consider the sample material of the swabs, as any organic material (e.g. cotton) would interfere with NGS analyses by increasing the sequencing background [19]. Furthermore, swabs should be sterile or autoclavable in order to allow for samples free of contamination. But also vice versa, the material was selected in order to be harmless for the health of the sampled bats.

Conclusion

We performed three individual bat sampling sessions to optimize sampling of multiple bat species roosting sympatrically in Wavul Galge cave, Sri Lanka. The samplings took place at different times of the year in order to adjust to different circumstances regarding bat population dynamics and colony size, breeding seasons and environment variables such as temperature and humidity. We compared different sampling setups and optimized the total duration of a sampling session, the sampling time point, the sampling itself and sample logistics in terms of storage and cold chain. All aspects were optimized in order to cause as little stress as possible for the sampled bats, to increase the safety of the research team and to obtain optimal sample output at the same time. The experiences gained during this research will help to perform frequent sampling sessions in the future and to monitor the wellbeing and conservation of this colony. The long-term observation will help to better understand zoological questions such as population dynamics and interaction of the different species. Molecular biological analyses and detection of viral shedding will help to link these ecological aspects to virological and public health-relevant questions. We expect that our observations and the subsequent optimization process for the sampling sessions will help other teams to plan or improve comparable bat sampling experiments in the context of One Health.

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One Health Approach for the sampling of different bat species living in a sympatric colony

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Supplementary Table

Table 1: Overview of material that was used as PPE and for catching and sampling of bats during the three conducted bat sampling sessions in Wavul Galge, Sri Lanka. If applicable, manufacturer and article details of the used equipment is indicated, but may be replaced by comparable articles.

Personal protective equipment (PPE)		
Description	**Application**	**Manufacturer, article details (if applicable)**
Baseball cap or hat	Head protection from bat droppings	Any suitable head protection
FFP3 mask	Protection from aerosols	3M Company, Saint Paul, Minnesota, USA Aura™ 9330+ (Article 3M-ID 7000088812)
Work gloves	Transfer of bats after catching	Any, available in construction markets
Lab gloves	Handling / Sampling of bats	MICROFLEX® NeoTouch™ (Article 25-201)
PU cut protection gloves	Handling / Sampling of bats	Engelbert Strauss, GmbH & Co. KG, Biebergemünd, Germany (Article #7616407)
Bat Catching		
Description	**Application**	**Manufacturer, article details (if applicable)**
G7 Harp Trap	Trapping of bats	Bat Conservation and Management Inc., Carlisle, Pennsylvania, USA
Hand net	Trapping of bats	Self-made, suitable nets are also available in shops for angling accessories
Bat holding bats	Trapping of bats	NHBS GmbH, Bonn, Germany (Article #234513)
Bat sampling		
Description	**Application**	**Manufacturer, article details (if applicable)**
Minitip FLOQSwabs®	Suitable for oral swabs	Copan Diagnostics, CA, USA (Article 501CS01)
CleanFoam® Swabs, round shape, 3.8 mm	Preferred swab for taking oral and urine swabs	ITW Texwipe, Kernersville, NC, USA (Article TX741B)
CleanFoam® Swabs, spear shape, 2.5 mm max	Preferred swab for taking rectal swabs	ITW Texwipe, Kernersville, NC, USA (Article TX751B)
dialMax Vernier Dial Caliper	Taking forearm length measurements of bats	Wiha Werkzeuge GmbH, Schonach, Germany (Article 27082)
Light-Line Spring Scale	Weighing of bats	PESOLA Präzisionswaagen AG, Schindellegi, Switzerland (Article 10050)
Forceps	Collection of fecal pellets, collection of ectoparasites	Bürkle GmbH, Bad Bellingen, Germany Article (5386-0300)
Sterile cannulas (Sterican 30G, 0.3x12mm)	Taking blood from bats	B. Braun, Melsungen, Germany (Article 4656300)
Whatman® protein saver cards	Storage of collected blood	Cytiva, Marlborough, Massachusetts, USA (Article 10531018)
2 ml screw cap mini tubes	For storage of collected samples, sterile	Sarstedt AG & Co. KG, Nümbrecht, Germany (Article 72.694.306)
Va-Q-Tcon Cooling Boxes	Short-term storage of samples (1 – 2 days)	va-Q-tec AG, Würzburg, Germany (Article BC000102)
Dry shipper VOYAGEUR Plus	Freezing and long-term storage of samples	Cryopal, Bussy Saint Georges, France (Article VOYAGEUR20-2)
Nylon socks	Facilitated storage of samples in the dry shipper	Any available socks

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Article

Paramyxovirus Diversity within One Population of *Miniopterus fuliginosus* Bats in Sri Lanka

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Abstract: Bats are known as typical reservoirs for a number of viruses, including viruses of the family Paramyxoviridae. Representatives of the subfamily Orthoparamyxovirinae are distributed worldwide and can cause mild to fatal diseases when infecting humans. The research on Paramyxoviruses (PMVs) from different bat hosts all over the world aims to understand the diversity, evolution and distribution of these viruses and to assess their zoonotic potential. A high number of yet unclassified PMVs from bats are recorded. In our study, we investigated bat species from the families Rhinolophidae, Hipposiderae, Pteropodidae and Miniopteridae that are roosting sympatrically in the Wavul Galge cave (Koslanda, Sri Lanka). The sampling at three time points (March and July 2018; January 2019) and screening for PMVs with a generic PCR show the presence of different novel PMVs in 10 urine samples collected from *Miniopterus fuliginosus*. Sequence analysis revealed a high similarity of the novel strains among each other and to other unclassified PMVs collected from *Miniopterus* bats. In this study, we present the first detection of PMVs in Sri Lanka and the presence of PMVs in the bat species *M. fuliginosus* for the first time.

Keywords: bat Paramyxovirus; *Miniopterus fuliginosus*; Sri Lanka

1. Introduction

The virus family Paramyxoviridae (PMV) belongs to the order Mononegavirales and represents large, enveloped viruses with a negative-sense ssRNA genome [1]. The family is further divided into several subfamilies, from which the Orthoparamyxovirinae is divided into eight genera. Representative viruses of these genera are able to infect a broad range of hosts, including reptiles, birds, fish and mammals [2]. Within the Orthoparamyxovirinae, human pathogenic viruses are described in the genera Respirovirus (e.g., human respirovirus 1 and 3), Morbillivirus (e.g., Measles virus) and Henipavirus (e.g., Hendra virus, Nipah virus) [3]. Bats within the order Chiroptera are known to host a high variety of viruses, including zoonotic viruses. Their ability to carry the viruses without suffering from viral infections makes them a research topic of high interest with respect to immune

response [4]. It is assumed that this is a result of a long-term co-evolution of viruses and bats [5]. Unique features of these mammals, such as their ability to fly, their migration patterns over long distances and their roosting habits in large colonies, promote them as suitable reservoirs for viruses [6]. Due to the intensified research on bat pathogens, the number of identified yet unclassified bat paramyxoviruses rose in the past decades [3]. Some zoonotic PMVs have a worldwide distribution; some highly pathogenic PMVs such as Nipah virus, Hendra virus and Menangle virus are recorded in Asia and Australia only [7]. Therefore, a regular screening and monitoring of viruses in bats and the characterization of novel PMVs can help to understand the evolution, distribution, zoonotic potential and transmission routes of these viruses.

With 31 recorded species, Chiroptera is a highly represented order of mammals in Sri Lanka [8]. Although bat species, their habitats, distribution and ecology are common research fields for zoologists in Sri Lanka, there are only a few studies available about their role as reservoirs for zoonotic viruses [9–12]. In our study, we examined samples from different bat species of the families Rhinolophidae, Hipposiderae, Pteropodidae and Miniopteridae, roosting together in one of the largest natural caves in Sri Lanka (Wavul Galge cave, Koslanda). In previous investigations, we reported alpha- and beta-coronaviruses in rectal swabs and feces samples of the two species *Miniopterus fuliginosus* and *Rousettus leschenaultii* [9]. Since all of the bat families inhabiting the cave were also known to carry PMVs, we aimed to screen all species for novel PMVs and concluded the zoonotic risk originating from the Wavul Galge cave. With this article, we present the molecular detection of different bat paramyxovirus strains for the first time in a population of *M. fuliginosus* bats and for the first time in Sri Lanka.

2. Results

In our study, we investigated a total of 143 urine samples that were collected at three sampling points in March and July 2018 and January 2019. An overview of the collected urine samples per sampling point and bat genus is shown in Table 1. Samples were obtained from the genera Hipposideros, Rhinolophus, Rousettus and Miniopterus. A majority of 102 urine samples from Miniopterus bats were collected during the sampling session in July 2018 and the remaining 11 were collected in January 2019. All collected samples were screened for paramyxoviruses using the semi-nested PCR assay and 10 urine samples from *M. fuliginosus* bats collected in July 2018 tested positive. De novo assembly of the Sanger sequences revealed the presence of three different PMV strains in the samples, sharing nucleotide (nt) identities between 80 and 81%.

Table 1. Overview of the urine samples (PMV-positive/total of samples) collected from different bat genera at three sampling points.

Genus	March 2018	June 2018	January 2019	Total Urine Samples
Miniopterus	0/0	10/102	0/11	**10/113**
Rousettus	0/2	0/2	0/6	**0/10**
Hipposideros	0/2	0/0	0/6	**0/8**
Rhinolophus	0/6	0/0	0/6	**0/12**

In the phylogenetic reconstruction (Figure 1), the three novel strains cluster monophyletically with a number of unclassified PMVs. Representatives of the genus Jeilongvirus build a paraphyletic branch close to the unclassified PMVs. The genera Henipavirus, Morbillivirus and Respirovirus appear as separate branches inside the clade of the Orthoparamyxovirinae subfamily. The selected representatives of Rubulavirinae and Avulavirinae subfamilies were reconstructed as clearly distinct clades.

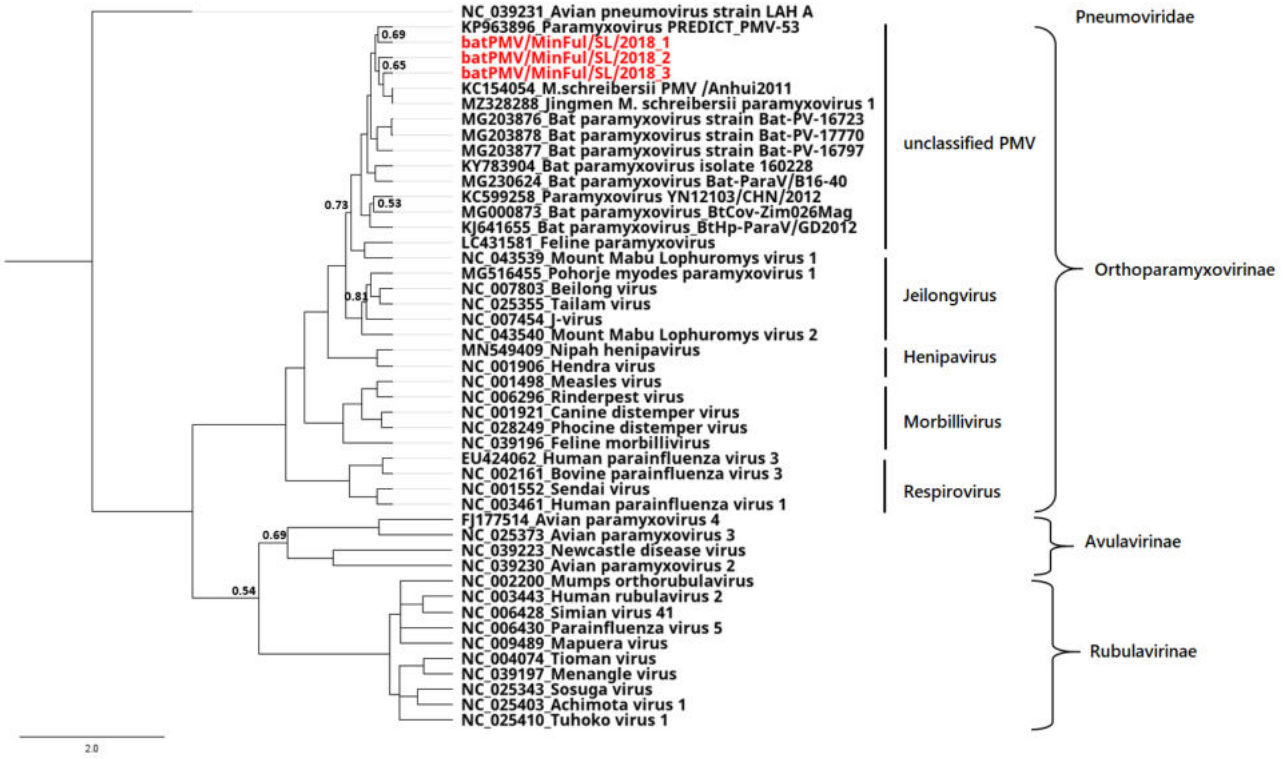

Figure 1. Phylogenetic tree based on a 473 nt alignment, sequence is located on the L Gene. The novel strains batPMV/MinFul/SL/2018_1–3 (red), selected PMV strains and representative Avulavirinae and Rubulavirinae were used for phylogenetic reconstruction. The avian pneumovirus strain LAH A (NC_039231) was included as an outgroup for the calculation. The phylogenetic tree was calculated with the Bayesian algorithm, and 1 million generations were calculated with a sub-sampling frequency of 100 and a burn-in of 10%. Substitution model GTR was selected with a gamma-distributed rate variation. Posterior probability values of <0.9 are shown next to the respective nodes.

The phylogenetic results are confirmed by a heatmap based on a multiple aa sequence alignment, including selected representative strains (Figure 2).

The Orthoparamyxovirinae share identities between 45.5% and 100% among each other. In general, high identities of the novel strains batPMV/MinFul/SL/2018_1–3 to other unclassified PMVs were calculated, ranging between 76.9 to 100%. The three novel strains share identities of 98.1% on aa level. High similarities were calculated to other bat PMVs and all novel strains share identities between 91 and 100% to other *Miniopterus* PMVs. The strain batPMV/MinFul/SL/2018_3 shares a 100% identity with two unclassified PMV strains (KC154054, MZ328288), which were both collected from *Miniopterus schreibersii* in China. In contrast, the novel strain batPMV/MinFul/SL/2018_2 shares a slightly lower identity of 96.8% to other strains from Sri Lanka.

The aa identities of the three novel strains to representatives from the genus Jeilongvirus are comparably high (71.2–76.9%). In contrast, they share lower identities to Henipaviruses (59–64.1%), Respiroviruses (55.1–60.3%) and Morbilliviruses (49.4–53.8%).

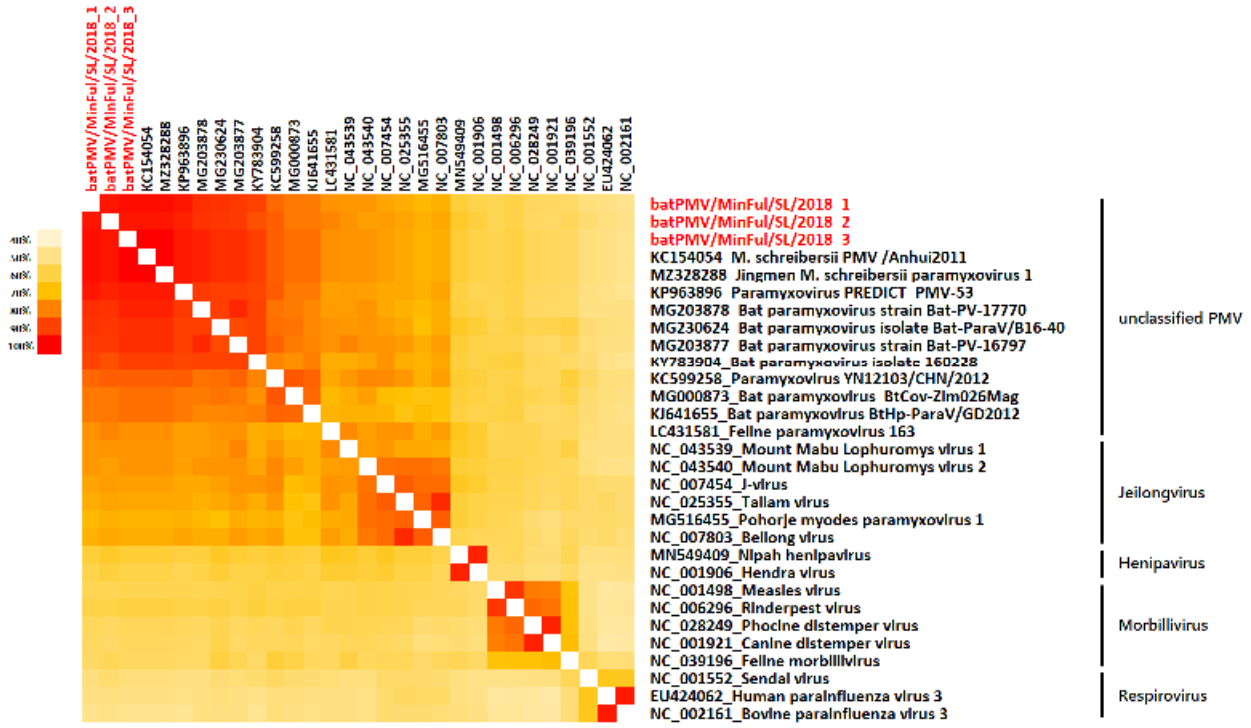

Figure 2. Heatmap based on a 156 aa alignment of the L gene coding for the RNA polymerase protein. The novel strains batPMV/MinFul/SL/2018_1–3 (red) and selected PMV strains belonging to the subfamily Orthoparamyxovirinae were used for alignment and heatmap calculation.

3. Discussion

These results demonstrate the first detection of PMVs in Sri Lankan bats and further the first detection within the species *M. fuliginosus* worldwide. In general, PMVs are known to be present on all populated continents [2,13]. Although PMVs can be found in a number of hosts such as reptiles, birds, fish and other mammals, only chiroptera-hosted PMVs are known to cause zoonotic diseases in humans [14].

Pteropodidae are natural hosts for henipaviruses, causing respiratory and neurological diseases in humans with high cases of fatalities [15]. On one hand, other PMVs of the genera Rubulavirus (mumps virus), Respirovirus (human respirovirus) and Morbillivirus (measles virus) are known to cause zoonotic diseases highly pathogenic to humans worldwide [14]. On the other hand, there is little knowledge about the increasing number of unclassified PMVs. A high number of these identified strains were detected in bats of the family Miniopteridae. In our phylogenetic reconstruction of the three novel PMV strains from Sri Lanka, batPMV/MinFul/SL/2018_1–3 were assigned to the same cluster of unclassified PMVs but also showed high similarities to other *Miniopterus* PMVs of the genus Jeilongvirus. The phylogenetic reconstruction is based on a 473 nt sequence fragment of the L gene. In general, this highly conserved area coding for the RdRP gene is suitable for phylogenetic analyses, though a reliable classification of the novel strains can only be performed with the complete sequence of the L gene according to ICTV classification criteria [1]. The massive diversity of the family Paramyxoviridae results in difficulties regarding a proper classification system since the establishment of the order Mononegavirales in 1991. Until today, this order was emended 10 times; the last ICTV update was released in 2019 [16,17]. Still, the general demarcation criterion of RdRP nucleotide sequences seems to be insufficient for proper classification. Other criteria, such as host range and host-specific receptor binding proteins (RBPs), biological context, pairwise analysis of sequence complementarity (PASC) for all ORFs, and the size of the P gene, are suggested to classify PMVs properly [18]. In return, this would increase the complexity of PMV classification significantly. Recent studies proposed Shaanvirus as a novel genus within the

Orthoparamyxovirinae subfamily [19,20]. The concerned PMVs were first isolated in Korea from *Miniopterus schreibersii* bats and characterized using the above-mentioned criteria. In our analyses, we included Shaanvirus sequences and found a high similarity to the three novel PMV strains from Sri Lanka. Deeper sequence analyses of the whole P gene and genome sequences will be necessary to allow for reliable statements and species proposals.

For instance, the investigation of the RBP Hemagglutinin (H), Hemagglutinin/Neuraminidase (HN) or attachment glycoprotein (G) are important motifs for the receptor-specific entry to the host cells and can give information about the zoonotic potential of the virus.

In general, bats from the genus Miniopterus were found to host a number of PMVs all over the world. In our study, we investigated bats from Wavul Galge, a sympatric cave for bats from the genera Miniopterus, Rhinolophus, Hipposideros and Rousettus. Although all of these bat genera are known to host PMVs, we detected the novel PMV strains only in the *M. fuliginosus* species [3]. This may be an indication of a host specificity of the novel PMVs to *M. fuliginosus*, which in return may be a result of the presumed co-speciation of the virus and the bat species [5]. Our comparative sequence analyses support this assumption, showing high similarities of PMVs that were found in bats of the genus Miniopterus. However, the number of collected urine samples from the other bat species in the cave is too low to make a final statement about host specificity.

Paramyxoviruses such as Hendra and Nipah viruses show a seasonal shedding pattern, which is presumably influenced by environmental factors [21]. The presented results suggest a seasonality of the novel PMVs as well. We found PMVs only in July 2018; at that point of the year, *M. fuliginosus* used the Wavul Galge cave as pre-maternity location and was overrepresented [22–24]. Consequently, we collected a significantly high number of urine samples from *M. fuliginosus* during this sampling, resulting in a 9.8% positive rate for the novel PMV strains in *M. fuliginosus* bats. At other sampling times, we could not detect PMVs in any of the bat genera inhabiting the cave, though the number of collected urine samples was comparably low at these sampling points. It may be possible that other bat species in the cave carry PMVs as well but show different shedding patterns. Therefore, further samplings of all bat species at different time points over the year could help to understand the seasonality of the novel PMV strains and investigate the presence of PMVs in other bat species inhabiting the cave. Further investigation of all collected samples with different virus detection methods can be used to obtain a comprehensive picture of the prevalence of PMV. Successful virus isolation and NGS analyses may reveal more viral sequence information and allow for further classification of the novel PMVs.

4. Materials and Methods

The investigative research on Sri Lankan bats was approved by the local governmental authority (Department of Wildlife Conservation, Sri Lanka, permit No. WL/3/2/05/18, issued 10 January 2018) and conducted in accordance with relevant guidelines and regulations. Bat sampling from cave-dwelling bats roosting in the Wavul Galge cave (Koslanda, Sri Lanka) was performed in March and July 2018 and January 2019 [9,12]. During the sampling procedures, different sample types were taken if available (feces or rectal swabs, oral swabs, blood, urine swabs). The urine swab samples were taken directly from the bat if available and snap-frozen in liquid nitrogen before storage at −80 °C. For further processing, 500 µL of sterile PBS were added to the urine swab and mixed by vortexing. RNA was extracted using the Viral RNA Mini Kit (QIAGEN, Hilden, Germany). The paramyxovirus screening was performed with a semi-nested PCR assay, targeting an overall 662 nt sequence (first round) on the highly conserved RNA polymerase L gene of paramyxoviruses [25]. The original protocol was slightly adapted, using the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Waltham, MA, USA) for the first round of the nested PCR assay and 300 nmol each of forward and reverse primer. A total of 2 µL of extracted RNA were used in a final reaction volume of 25 µL. The PCR mixture was incubated at 45 °C for 15 min for the reverse transcription step, followed by an initial

denaturation step at 95 °C for 10 min. Forty cycles of PCR were performed at 95 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s, followed by a final incubation step at 72 °C for 6 min.

For the second round of the semi-nested PCR assay a Platinum Taq DNA Polymerase Kit (Invitrogen, Carlsbad, CA, USA) was used. For each reaction, 300 nmol of forward and reverse primer, 2.5 mM MgCl2, 250 µM dNTPs, 1 × Platinum Taq Buffer and 1.25 U of Platinum Taq DNA polymerase were used. Water was added to a final volume of 23 µL and 2 µL of the first-round PCR product were added. Thermal cycling of the second PCR round was initialized with a denaturation at 95 °C for 2 min, followed by 45 cycles at 95 °C for 15 s, 50 °C for 30 s, 72 °C for 30 s and a final incubation at 72 °C for 6 min.

Products of both PCR rounds were run and analyzed simultaneously on a 1.5% agarose gel containing DNA Stain G (SERVA, Heidelberg, Germany). Samples showing a distinct band in the gel analysis were purified by using MSB Spin PCRapace Kit (Invitrogen, Carlsbad, CA, USA) and sequenced with a BigDye Terminator Cycle Sequencing Kit on a 3500 Dx Genetic Analyzer (Applied Biosystems, Waltham, MA, USA), using the corresponding forward and reverse primers for each strand.

Sanger sequences were analyzed and checked for quality by using Geneious Prime software, low-quality bases at the end of each sequence were trimmed before further processing.

A de novo assembly of all sanger sequences was calculated using Velvet assembler [26]. The resulting sequences are available online at GenBank and are named batPMV/MinFul/SL/2018_1 (Accession number OM777173), batPMV/MinFul/SL/2018_2 (Accession number OM777172) and batPMV/MinFul/SL/2018_3 (Accession number OM777171).

A nucleotide alignment of 473 nt was calculated by using MAFFT algorithm v7.450 [27]. The alignment contained the three novel strains from Sri Lanka as well as different PMV representative strains from different Orthoparamyxovirinae genera downloaded from the NCBI database. A phylogenetic tree was calculated by using MrBayes version 3.2.6 [28]. The model GTR with gamma-distributed rate variation was selected for these calculations; parameters were set as follows: number of runs: four; number of generations: 1,000,000; subsampling frequency: 100; and burn in: 10%. The reference strain avian pneumovirus LAH A (NC_039231) was selected as outgroup for the calculations. The phylogenetic tree was visualized with Geneious Prime software.

5. Conclusions

With this study, we detected PMVs for the first time in *M. fuliginosus* and for the first time in Sri Lanka. Sequence analysis revealed the presence of different strains with high similarity to other unclassified PMVs. The results indicate a high host-specificity of the novel strains batPMV/MinFul/SL/2018_1–3 to the bat species *M. fuliginosus*; we did not detect the novel strains in other bat species inhabiting the cave. Studying the sympatric bat species in Wavul Galge, one of the largest caves in Sri Lanka, can give important insights to virus–host interaction, host specificity and the seasonal shedding of viruses. Therefore, we suggest a long-term monitoring of the bats and their viruses in the cave, whereby a respectful interaction is important to protect their natural habitat.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 1 March 2022, Accession number OM777171–OM777173).

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Detection of Alpha- and Betacoronaviruses in *Miniopterus fuliginosus* and *Rousettus leschenaultii*, two species of Sri Lankan Bats

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Abstract: Bats are known to be potential reservoirs of numerous human-pathogenic viruses. They have been identified as natural hosts for coronaviruses, causing Severe Acute Respiratory Syndrome (SARS) in humans. Since the emergence of SARS-CoV-2 in 2019 interest in the prevalence of coronaviruses in bats was newly raised. In this study we investigated different bat species living in a sympatric colony in the Wavul Galge cave (Koslanda, Sri Lanka). In three field sessions (in 2018 and 2019), 395 bats were captured (*Miniopterus*, *Rousettus*, *Hipposideros* and *Rhinolophus* spp.) and either rectal swabs or fecal samples were collected. From these overall 396 rectal swab and fecal samples, the screening for coronaviruses with nested PCR resulted in 33 positive samples, 31 of which originated from *Miniopterus fuliginosus* and two from *Rousettus leschenaultii*. Sanger sequencing and phylogenetic analysis of the obtained 384-nt fragment of the RNA-dependent RNA polymerase revealed that the examined *M. fuliginosus* bats excrete alphacoronaviruses and the examined *R. leschenaultii* bats excrete betacoronaviruses. Despite the sympatric roosting habitat, the coronaviruses showed host specificity and seemed to be limited to one species. Our results represent an important basis to better understand the prevalence of coronaviruses in Sri Lankan bats and may provide a basis for pursuing studies on particular bat species of interest.

Keywords: bat coronavirus; *Miniopterus fuliginosus*; *Rousettus leschenaultii*; Sri Lanka; cave-dwelling; sympatric colony; alphacoronavirus; betacoronavirus

1. Introduction

Bats (*Chiroptera*) are an order of mammals with the highest variety of species worldwide [1]. In Sri Lanka, the suborders of *Yinpterochiroptera* and *Yangochiroptera* are both represented in high numbers, accounting for almost 1/3 of the Sri Lankan mammals with 30 different species [2]. Their species variety and other unique features, specifically their ability to fly long distances, their migratory behavior and gregarious roosting habits, make them important virus reservoirs [1]. The study of these unique animal hosts is an important field of virology in order to learn more about the co-evolution of bats and viruses and the potential zoonotic transmission of viruses from bats to humans. Furthermore, an extensive

knowledge about viral transmission mechanisms and virus-host interactions facilitates the development of antiviral drugs and vaccines. In the past decades, a number of zoonotic events has been resulting in an increase of emerging infectious diseases. Outbreaks of Ebola and Marburg viruses, Hendra virus, Nipah virus and coronaviruses (SARS-CoV and SARS-CoV-2, MERS-CoV) are among the most prominent examples [3–5]. Coronaviruses (CoV) are a family of RNA viruses that can be subdivided into the genera of alpha- (α-CoV), beta- (β-CoV), gamma- (γ-CoV) and delta-coronaviruses (δ-CoV) [6]. So far, predominantly α-CoVs and β-CoVs were detected in bat species which may be their natural reservoir, although they can also be found in other domestic animals such as swine and horses or in wild animals such as donkeys [7]. Most probably, γ-CoVs and δ-CoVs derived from bird CoVs. Different CoVs such as HCoV-NL63 and HCoV-229E (α-CoV), HCoV-OC43 and HKU1 (β-CoV) cause mild respiratory symptoms in humans [8]. The group of β-CoVs also includes virus species which can cause severe respiratory symptoms and which have the potential to spread rapidly and easily among humans, demonstrated in the past and current pandemics of SARS-CoV, MERS-CoV and SARS-CoV-2. Especially under these circumstances, the understanding of bats and their role as reservoirs of certain viruses has become of special interest and is being enhanced in numerous Asian countries [9–11]. Sri Lanka has a rich biodiversity, also reflected by the presence of 30 different bat species inhabiting the island [12]. With such a high diversity, it can be assumed that α- and β-CoVs would be present in Sri Lankan bat populations, as bats are major hosts of these viruses. Already in 2018, Kudagammana et al. have detected CoV in flying foxes (*Pteropus medius*) in Sri Lanka [13]. Our research was focused on a population of bats in the Wavul Galge cave (Koslanda, Sri Lanka), one of the largest natural caves in Sri Lanka which is permanently occupied by five species of bats. With this study we expand the evidence of α- and β-CoVs in the two cave-dwelling Sri Lankan bat species *R. leschenaultii* and *M. fuliginosus*.

2. Materials and Methods

Investigative research on Sri Lankan bats was approved by the local governmental authority (Department of Wildlife Conservation, Sri Lanka) and conducted in accordance with relevant guidelines and regulations. Samples from Sri Lankan cave-dwelling bats roosting in the Wavul Galge cave were taken at three different points in time (March and July 2018 and January 2019). A total of 395 bats belonging to the genera *Miniopterus*, *Rousettus*, *Rhinolophus* or *Hipposideros* were captured and kept in bat holding bags until further processing to avoid sampling one individual twice. Sampling of the bats was performed while using adequate personal protection equipment, namely safety gloves, safety glasses and FFP3 masks. The bat species was determined macroscopically and documented. Additionally, oral swabs were taken from each bat for molecular species identification based on the cytochrome B gene [14]. Either rectal swabs were taken with sterile swabs, or feces was collected with forceps from the bat holding bags if droppings were available. Swabs and fecal samples were collected in tubes without any additives and stored natively in liquid nitrogen for transportation. For further processing, 500 µL of sterile PBS were added to rectal swabs or fecal samples. Rectal swab samples were mixed by vortexing, and fecal samples were homogenized by using ceramic beads and the FastPrep-24 device (MP Biomedicals, Eschwege, Germany). After a centrifugation step the supernatants were collected and used for RNA extraction with the Viral RNA Mini Kit (QIAGEN, Hilden, Germany).

Extracted RNA was transcribed to cDNA with a random hexamer primer by using SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The coronavirus screening was performed with a nested PCR assay [15] which is designed on the highly conserved RNA-dependent RNA polymerase (RdRP) gene of the coronavirus genome and amplifies a product of 455 bp. The original protocol was slightly adapted as follows, using cDNA instead of RNA as sample material and Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) for PCR amplification. For the first round of the nested PCR assay, mixtures contained 300 nM of primer PC2S2

and 900 nM of primer PC2AS1, 2.5 mM $MgCl_2$, 250 µM dNTPs, 1x Platinum Taq Buffer and 0.5 U of Platinum Taq DNA polymerase. Water was added to a final volume of 23 µL and 2 µL of cDNA were added per reaction. Positive and negative controls were included in each PCR set up to validate the results. The thermal cycling of the PCR was performed as described in the original protocol [15]. For the second round of the nested PCR assay, mixtures contained 300 nM of primer PCS and 400 nM of primer PCNAs; all other reagents were used in the same concentrations as in the first round. Water was added to a final volume of 23 µL and 2 µL of the first-round PCR product were added. Thermal cycling of the second PCR round was performed as described in the original protocol [15].

Products of both PCR rounds were run and analyzed simultaneously on a 1.5% agarose gel containing DNA Stain G (SERVA, Heidelberg, Germany). Positive PCR products were purified by using MSB Spin PCRapace Kit (Invitrogen, Carlsbad, CA, USA) and sequenced with a BigDye Terminator Cycle Sequencing Kit on an Applied Biosystems 3500 Dx Genetic Analyzer, using the corresponding forward and reverse primers for each strand.

Sanger sequences were analyzed by using Geneious Prime software, and low-quality bases at the end of each sequence were trimmed before further processing. A nucleotide alignment of 384 nt was calculated by using MAFFT algorithm v7.450 [16]; the alignment contained all sequences of the positive samples as well as CoV reference strains obtained from the NCBI database. Editing was performed with MEGA7. A phylogenetic tree was calculated by using MrBayes version 3.2.6 [17]. The model HKY85 with gamma-distributed rate variation was selected for these calculations; parameters were set as follows: number of runs: four; number of generations: 10,000,000; subsampling frequency: 10 and burn-in: 50%. The reference strain avian infectious bronchitis virus (NC_001451, ICTV type species for γ-CoVs) was selected as outgroup for the calculations. The phylogenetic tree was visualized with the Geneious Prime software.

3. Results

In total, 255 rectal swabs and 141 fecal samples from different bat species were tested by using the adapted nested PCR protocol for the generic detection of coronaviruses (Table 1).

Table 1. Overview of rectal swabs and fecal samples (CoV-positive/total of samples). Results are listed per bat genus and sampling session.

Genus	March 2018		June 2018		January 2019		Sampled Bats in Total
	Rectal swabs	Feces	Rectal swabs	Feces	Rectal swabs	Feces	
Miniopterus	0/3	0/0	20/115	5/76	0/4	6/27	31/225
Rousettus	0/8	0/2	2/11	0/0	0/16	0/3	2/40
Hipposideros	0/3	0/0	0/1	0/0	0/16	0/7	0/27
Rhinolophus	0/62	0/9	0/0	0/0	0/16	0/17	0/104
Total samples per session	76	11	127	76	52	54	

A total of 33 samples were tested positive (Table 2). The positive samples of this study were named after the corresponding bat species, the sampling session dates (March 2018 = 03-18, July 2018 = 07-18, January 2019 = 01-19) and the internal sample number. For example, the positive rectal swab (RS) sample RS170 from a *M. fuliginosus* bat collected in July 2018 was named batCoV/MinFul/07-18/RS170.

Table 2. Details on the positive rectal swabs and fecal samples. The table lists the bat species determined by cytB sequencing (sequence data available on request), the sampling date, sex and forearm length of the sampled bats, the given name of the detected CoV and its GenBank accession number. All bats were captured and sampled in the Wavul Galge cave, Koslanda, Sri Lanka. n.a. = not applicable.

Sample	Species	Date	Sex	Forearm Length (cm)	Bat CoV Description	Accession Number
RS85	*M. fuliginosus*	07/07/18	m	4.76	batCoV/MinFul/07-18/RS85	MW987547
RS90	*R. leschenaultii*	07/07/18	f	5.67	batCoV/RousLesch/07-18/RS90	MW987539
RS91	*M. fuliginosus*	07/07/18	f	4.55	batCoV/MinFul/07-18/RS91	MW987548
RS94	*M. fuliginosus*	08/07/18	m	4.53	batCoV/MinFul/07-18/RS94	MW987549
RS96	*M. fuliginosus*	07/07/18	f	4.49	batCoV/MinFul/07-18/RS96	MW987554
RS106	*M. fuliginosus*	07/07/18	f	4.54	batCoV/MinFul/07-18/RS106	MW987555
RS112	*R. leschenaultii*	07/07/18	f	n.a.	batCoV/RousLesch/07-18/RS112	MW987540
RS114	*M. fuliginosus*	07/07/18	f	4.65	batCoV/MinFul/07-18/RS114	MW987550
RS118	*M. fuliginosus*	07/07/18	f	4.65	batCoV/MinFul/07-18/RS118	MW987556
RS124	*M. fuliginosus*	07/07/18	f	4.65	batCoV/MinFul/07-18/RS124	MW987546
RS158	*M. fuliginosus*	08/07/18	f	4.41	batCoV/MinFul/07-18/RS158	MW987566
RS170	*M. fuliginosus*	08/07/18	f	4.91	batCoV/MinFul/07-18/RS170	MW987545
RS172	*M. fuliginosus*	08/07/18	f	4.64	batCoV/MinFul/07-18/RS172	MW987552
RS187	*M. fuliginosus*	08/07/18	f	4.61	batCoV/MinFul/07-18/RS187	MW987563
RS190	*M. fuliginosus*	08/07/18	m	4.65	batCoV/MinFul/07-18/RS190	MW987559
RS193	*M. fuliginosus*	08/07/18	f	4.58	batCoV/MinFul/07-18/RS193	MW987560
RS198	*M. fuliginosus*	08/07/18	f	4.54	batCoV/MinFul/07-18/RS198	MW987564
RS199	*M. fuliginosus*	08/07/18	f	4.51	batCoV/MinFul/07-18/RS199	MW987542
RS236	*M. fuliginosus*	09/07/18	f	4.67	batCoV/MinFul/07-18/RS236	MW987543
RS276	*M. fuliginosus*	10/07/18	f	4.45	batCoV/MinFul/07-18/RS276	MW987553
RS277	*M. fuliginosus*	10/07/18	m	4.65	batCoV/MinFul/07-18/RS277	MW987561
RS278	*M. fuliginosus*	10/07/18	f	4.41	batCoV/MinFul/07-18/RS278	MW987557
F95	*M. fuliginosus*	07/07/18	f	4.38	batCoV/MinFul/07-18/F95	MW987541
F128	*M. fuliginosus*	07/07/18	m	4.55	batCoV/MinFul/07-18/F128	MW987562
F142	*M. fuliginosus*	07/07/18	f	4.69	batCoV/MinFul/07-18/F142	MW987544
F153	*M. fuliginosus*	07/07/18	m	4.59	batCoV/MinFul/07-18/F153	MW987568
F155	*M. fuliginosus*	08/07/18	f	n.a.	batCoV/MinFul/07-18/F155	MW987567
F334	*M. fuliginosus*	23/01/19	m	4.65	batCoV/MinFul/01-19/F334	MW987565
F347	*M. fuliginosus*	23/01/19	f	4.76	batCoV/MinFul/01-19/F347	MW987569
F350	*M. fuliginosus*	23/01/19	f	4.51	batCoV/MinFul/01-19/F350	MW987571
F351	*M. fuliginosus*	23/01/19	m	4.55	batCoV/MinFul/01-19/F351	MW987558
F353	*M. fuliginosus*	23/01/19	m	4.58	batCoV/MinFul/01-19/F353	MW987570
F356	*M. fuliginosus*	23/01/19	m	4.56	batCoV/MinFul/01-19/F356	MW987551

Sanger sequencing of the positive samples revealed a consensus sequence of at least 384 nucleotides. The sequences of batCoV/MinFul/07-18/RS91 and batCoV/MinFul/07-18/RS94 showed 100% identity, as did the sequences batCoV/MinFul/07-18/RS114 and batCoV/MinFul/01-19/F356. All other samples were unique in the 384 nt sequence with at least one nucleotide difference to the others (with identities ranging from 65% to 99%). This is visualized by an alignment-based nucleotide heatmap of all Sri Lankan bat samples (Figure 1). Twenty-seven of the *M. fuliginosus* sequences (both rectal swabs and fecal samples) have a high identity of 100–95%. Further four sequences have an identity of less than 80–75% to the other samples, while three of them (batCoV/MinFul/07-18/F153, -01-19/F347 and -01-19/F353) share a high identity of 98–99% among each other. Only the sample batCoV/MinFul/07-18/F350 has a lower identity of 77% to the three sequences and the lowest identity of 75–76% to all other *M. fuliginosus* sequences.

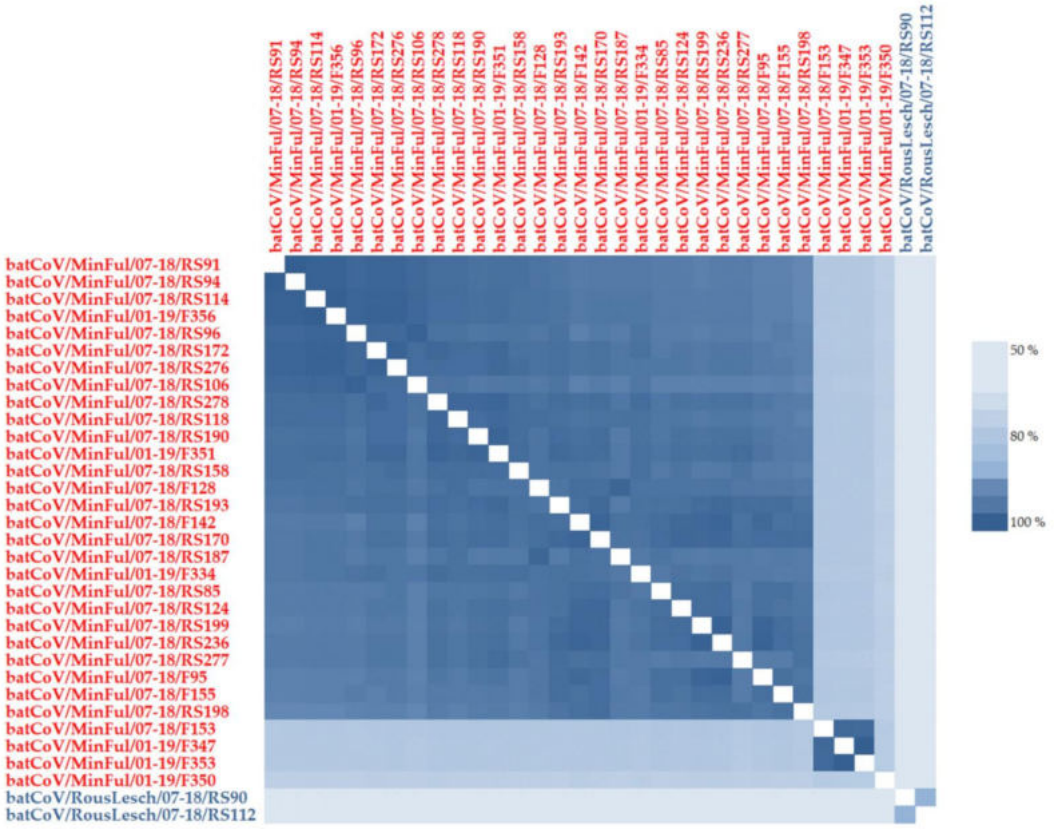

Figure 1. Heatmap based on a nucleotide alignment of 384 bp PCR product of the RdRP gene of the coronavirus genome. The figure illustrates the identities of all CoV-positive rectal swabs or fecal samples collected in the Wavul Galge cave, Koslanda, Sri Lanka, at three different points in time. Red sequences represent positive M. fuliginosus bats, whereas blue sequences indicate positive R. leschenaultii bats.

Apart from this, the two positive samples from *R. leschenaultii* bats share an identity of 56–63% to the *M. fuliginosus* sequences. Among each other, the sequence identity is 90%.

Furthermore, a phylogenetic tree was calculated with all sequences obtained from this study and other CoV reference strains from the NCBI database (Figure 2). In general, all *M. fuliginosus* sequences were allocated to the branch of α-CoV. The 29 highly identical sequences (95–100% identity) form a separate phylogenetic clade with three further *Miniopterus* spp. sequences from China and Hongkong [18,19]. The four *M. fuliginosus* sequences showing a lower identity on nucleotide level (Figure 1) were allocated to different groups in the phylogenetic tree. While three of them cluster with a *Miniopterus* spp. batCoV HKU8 strain, the least identical sequence (batCoV/MinFul/07-18/F350) forms a common clade with a *Miniopterus* spp. batCoV HKU7 strain. Human CoV-like 229E and NL63 are assigned also to the α-CoVs but have a higher distance to bat CoV strains.

Both sequences from *R. leschenaultii* samples were allocated to the branch of β-CoV. Within this branch, both sequences form a small group with two other β-CoVs from *Rousettus* spp. sampled in India [20]. Other β-CoVs like HCoV OC43 and SARS-CoV-2 strains from China and Sri Lanka form separate groups and have a higher distance from this branch.

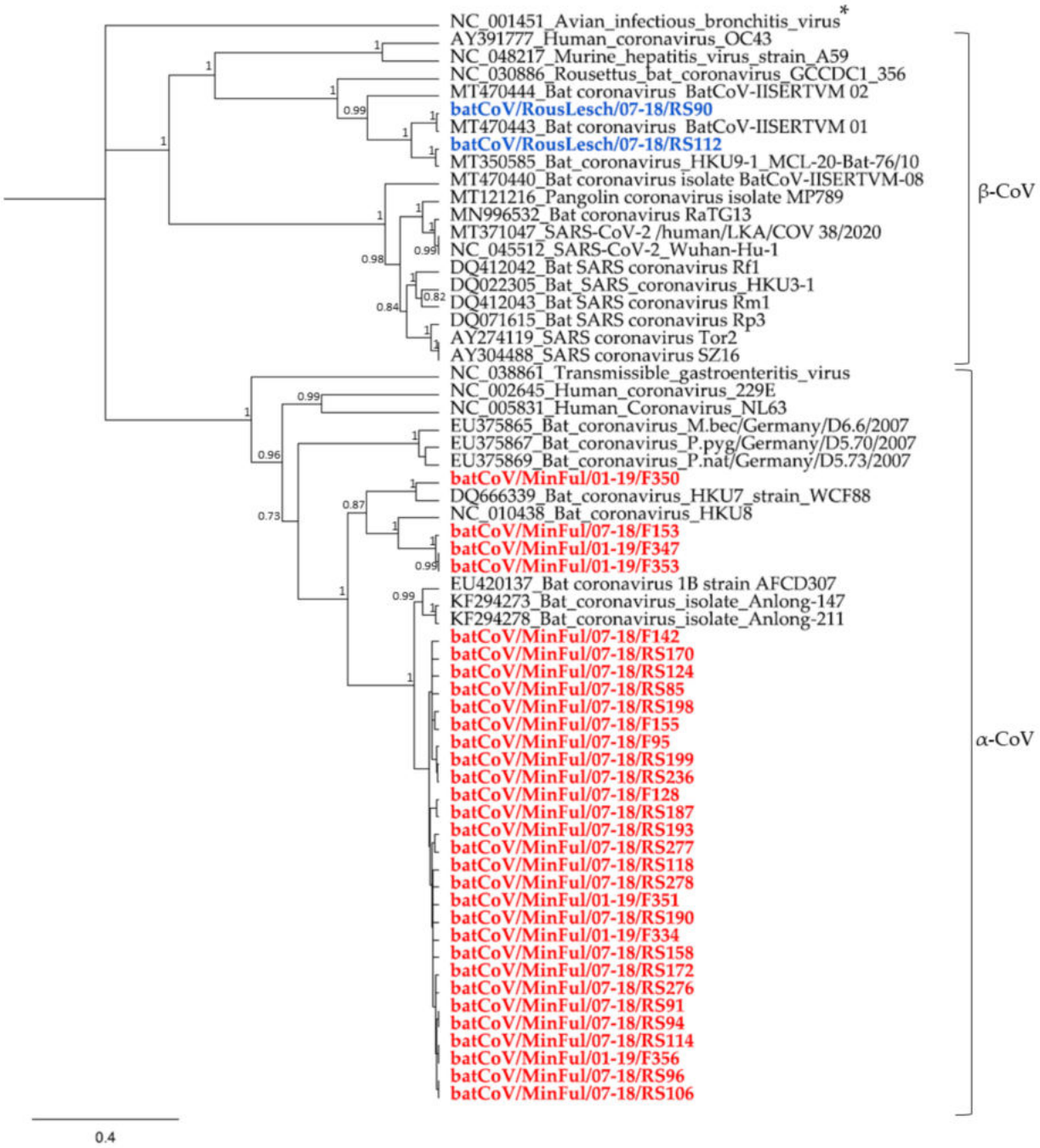

Figure 2. Phylogenetic tree of coronavirus sequences from Sri Lankan M. fuliginosus bats (given in red) and R. leschenaultii bats (given in blue) and other reference sequences of different α- and β-CoVs. The tree is divided into the two groups of α- and β-CoVs. In addition, the γ-CoV avian infectious bronchitis virus (NC_001451, marked with an asterisk) was included as outgroup for the calculation. The phylogenetic tree was calculated with Bayesian algorithm, and 5 mio trees were calculated with a subsampling frequency of 25 and a burn-in of 50%. Substitution model HKY85 was selected with a gamma-distributed rate variation. Branch label values for the 27 bat CoV sequences that form a joint clade are not displayed in the tree; their posterior probability values range between 0.52 and 1.

4. Discussion

In this study we detected α- and β-CoVs in *R. leschenaultii* and *M. fuliginosus* bats in Sri Lanka for the first time. Only in 2018, another study found β-CoVs in Sri Lankan flying foxes (*Pteropus medius*), but since there was no access to the sequences we were not able to include them in our phylogenetic analysis for comparison [13].

4.1. α-CoVs in Miniopterus fuliginosus Bat Samples

In total, we found 31 α-CoVs from *M. fuliginosus* rectal swabs and fecal samples. The focus of the bat sampling in our study was on *M. fuliginosus* bats, mainly because of their migration behavior. Therefore, we collected the highest number of samples from this species during all three sampling sessions. In return, most of our samples that were screened positive were obtained from *M. fuliginosus* bats. A first comparison in a nucleotide alignment and the corresponding heatmap shows that the sequences have a high variety among each other. Even the first cluster of 29 samples partially reveals identities of 97% and less which can be considered low for this short sequence on the highly conserved RdRP gene. The structural functionality of the RdRP gene is essential for viral replication; this gene is therefore less susceptible to mutation events than other parts of the genome [21]. Despite this, RNA viruses like CoVs replicate with a mutation rate of one per 1000 to 10,000 nucleotides [22], which results in the development of novel coronaviruses and also explains a high diversity of the RdRP gene on nucleotide level. The separation of the *M. fuliginosus* sequences into at least two clusters therefore indicates the presence of different α-CoVs within the species. The phylogenetic analysis supports the assumption that different viral strains are present which all belong to the group 1 α-CoVs [22]. The related CoV HKU8 and CoV HKU7 strains in that cluster were obtained from *Miniopterus* spp. as well. As reported elsewhere before, different bat species from the genus *Miniopterus* probably serve as hosts for HKU7, HKU8 and closely related CoVs [18]. The presence of multiple viral strains within the *M. fuliginosus* cave population would be reasonable, considering the fact that these bats migrate seasonally from small surrounding colonies to the Wavul Galge cave, using it as a pre-maternity cave [2]. The migration time is between July and August and matches our second sampling session (July 2018), when we found most of the positive samples (25 α-CoVs in *M. fuliginosus* bats, see Table 1) [12]. Another explanation might also be that 65% of the positive samples were from female *M. fuliginosus* bats. In general, the number of female bats was increased at that sampling point, as 85% of the sampled *M. fuliginosus* bats in July 2018 were females. Persistence and circulation of different α-CoV strains in the small neighboring colonies could be assumed, and further studies would be necessary to investigate whether transmission or exchange of these different virus strains within the *M. fuliginosus* species occurs when they migrate to the Wavul Galge cave.

4.2. β-CoVs in Rousettus leschenaultii Bat Samples

In addition to the detected α-CoVs in *M. fuliginosus* samples, we found two β-CoVs in *R. leschenaultii* bat samples. *R. leschenaultii* is a fruit bat and the only cave-dwelling megachiropteran species [12]. In contrast to the *M. fuliginosus* species, they do not show seasonal migration behavior but are long-term inhabitants of the Wavul Galge cave. Both positive samples were taken during the same session (July 2018); the low sequence identity of 90% suggests that we detected two different β-CoVs strains that probably persist in the *R. leschenaultii* population of the Wavul Galge cave. A follow-up study with a higher number of samples from *R. leschenaultii* bats could prove whether the virus can be found in more *R. leschenaultii* bats or whether even more β-CoVs strains are present in this species.

The phylogeny of both sequences allocates them to other β-CoVs of the HKU9 strain. *Rousettus* spp. in India [20], China [23,24] and Singapore [25] already tested positive for HKU9 β-CoVs and may be their natural reservoir.

4.3. Presumed Host Specificity of Bat CoVs

Apart from the presented results, the sampled *Rhinolophus* spp. and *Hipposideros* spp. were not tested positive for any CoVs. Generally speaking, the number of sampled bats was probably insufficient to make a final statement, and further sampling with an increased number of bats should follow to study the prevalence of CoV in these species more thoroughly. Another aspect is the shedding of viruses in some bat species, possibly influenced by seasonal changes and environmental conditions [26,27]. We collected samples in January, March and July. Sampling *Rhinolophus* spp. and *Hipposideros* spp. at other

points in time might clarify whether these species shed CoVs in other seasons of the year. Interestingly, the prevalence of CoV in the Wavul Galge cave seems to be very host specific, although *M. fuliginosus*, *R. leschenaultii*, *Rhinolophus* spp. and *Hipposideros* spp. roost sympatrically in the cave. For example, we could only detect α-CoVs in *M. fuliginosus* bats, although *Hipposideros* spp. are also known to carry α-CoVs and are assumed to be the natural host of human CoV 229E [28]. Furthermore, *Rhinolophus* spp. are known to carry SARS-like β-CoVs and may be the natural reservoir of the pandemic SARS-CoV-2 [10]. Although transmission of CoVs is generally possible [10,29], it did not seem to occur in this sympatric colony. One reason may be the spatial separation of the different species inside the Wavul Galge cave; therefore, direct contact between the species is only likely when entering or exiting the cave. Still, the probability of aerosol-based transmission between the species in the cave should be considered to be very high. Therefore, long-term monitoring of all bat species in the cave could help understand host specificity and transmission dynamics of these viruses.

4.4. Evaluating the Risk of Viral Spillover to Humans

As a result of the Covid-19 pandemic, in 2021, Grange et al. developed an open-source risk ranking tool to evaluate the risk of viral spillover to humans and the spreading potential of these viruses [30]. For their ranking, they selected innovative risk factors, divided into host risk factors, environmental risk factors and virus risk factors. With the help of this tool (https://spillover.global/ranking-comparison; accessed on 10 June 2021), we evaluated the spillover risk of the α-CoVs and β-CoVs we detected in *R. leschenaultii* and *M. fuliginosus* samples in Sri Lanka. For this purpose, we used the phylogenetic tree and selected the closest related strains. For *R. leschenaultii*, we checked the bat coronavirus HKU9 which has a high ranking score of 80 out of 155 and can be found in position 14 of the overall risk ranking of 887 viruses (https://spillover.global/virus/21; accessed on 10 June 2021). The risk factors of this *Rousettus* bat coronavirus HKU9 are for example the high host diversity (found in 11 bat species), the global distribution of the virus and the high interaction of wildlife, domestic animals and humans in these regions. For the α-CoVs from *M. fuliginosus* samples, we checked three of the closest related CoVs. The bat coronavirus 1 was the closest related strain for most of the α-CoVs from *M. fuliginosus* samples. With a risk score of 72 out of 155 it is ranked at position 40 (https://spillover.global/virus/49; accessed on 10 June 2021). The virus can be found in six different bat species, including three *Miniopterus* spp. and one species from the genera *Hipposideros*, *Myotis* and *Rhinolophus*, respectively. This host range may be of interest because *Hipposideros* and *Rhinolophus* bat species roost in the same cave, but were not tested positive for any CoVs so far. As discussed before, long-term monitoring may reveal CoVs in these species as well and could further support the host range given by the spillover ranking tool. Another important factor that has an impact on the spillover risk is the interaction of animals and humans in the region where the virus is found. The distribution of the virus is only semi-global and may explain its lower spillover risk. The Miniopterus bat coronavirus HKU8 can be found in position 107 with a spillover risk of 65 out of 155 (https://spillover.global/virus/114; accessed on 10 June 2021). It can only be found in *Miniopterus* spp. and is distributed semi-globally; in its distribution area, the interaction between humans and host animals is high. The same applies to the last bat coronavirus HKU7 which can be found semi-globally in two *Miniopterus* spp. and one *Taphozous* spp., while the interaction of the hosts and wild animals with humans is rated as medium. Consequently, this virus has the low position of 382 in the overall ranking with a risk score of 56 out of 155.

To sum up, this tool provides a good ranking system to evaluate the risk of spillover events to humans. The results confirm what has already been discussed before by assigning a rather low risk for zoonotic events to the α-CoVs detected in the *M. fuliginosus* species. In contrast, the β-CoVs from *R. leschenaultii* samples were ranked with a higher risk, which is reasonable considering the fact that other related β-CoVs from bat species are already known for their role in different spillover events.

The risk factors that are included in this ranking tool (virus, host and environmental factors) point out the high impact of the interaction of humans and wildlife which contributes significantly to the accumulation of zoonotic events.

5. Conclusions

With this study, we provide the first molecular biological analysis of different viruses in bat species roosting in the Wavul Galge cave, Sri Lanka. We detected α-CoVs in *M. fuliginosus* and β-CoVs in *R. leschenaultii* bats. Our results indicate that different virus strains are persistently present within both populations. Detailed gene and genome analysis of the existing CoVs as well as further studies with a focus on the other sympatric species (*Rhinolophus* spp. and *Hipposideros* spp.) might provide more insight into the prevalence, circulation or persistence of different CoV strains in this cave. A long-term study would be helpful to examine the seasonal shedding of the viruses and the impact of migration behavior of the different bat species. Our results indicate that the detected CoVs are host specific for the respective bat species and, despite the sympatric cohabitation in the Wavul Galge cave, an inter-species transmission was not observed. This supports the assumption that only particular bat species serve as natural reservoir for harmful human-pathogenic viruses like SARS-CoV, while spill-over events may occur because of environmental impact and the intrusion of humans to the living areas of the bat species [31]. Studying bats and monitoring their viruses as well as the respectful interaction with their natural habitats are both important factors to better understand and prevent zoonotic transmission from bats to humans. Being the largest cave in Sri Lanka with as many as five sympatric bat species, numbering over 100,000 individuals, the Wavul Galge cave provides an excellent natural site for long-term monitoring of bat-borne viruses in Sri Lanka. Our study emphasizes the need to periodically monitor all bat species and their viruses in this cave.

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Data Availability Statement: The data presented in this study are openly available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), Accession numbers MW987539–MW987571.

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Full Genome of batCoV/MinFul/2018/SriLanka, a Novel Alpha-Coronavirus Detected in *Miniopterus fuliginosus*, Sri Lanka

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Abstract: Coronaviruses (CoV) are divided into the genera α-CoVs, β-CoVs, γ-CoVs and δ-CoVs. Of these, α-CoVs and β-CoVs are solely capable of causing infections in humans, resulting in mild to severe respiratory symptoms. Bats have been identified as natural reservoir hosts for CoVs belonging to these two genera. Consequently, research on bat populations, CoV prevalence in bats and genetic characterization of bat CoVs is of special interest to investigate the potential transmission risks. We present the genome sequence of a novel α-CoV strain detected in rectal swab samples of *Miniopterus fuliginosus* bats from a colony in the Wavul Galge cave (Koslanda, Sri Lanka). The novel strain is highly similar to Miniopterus bat coronavirus 1, an α-CoV located in the subgenus of Minunacoviruses. Phylogenetic reconstruction revealed a high identity of the novel strain to other α-CoVs derived from Miniopterus bats, while human-pathogenic α-CoV strains like HCoV-229E and HCoV-NL63 were more distantly related. Comparison with selected bat-related and human-pathogenic strains of the β-CoV genus showed low identities of ~40%. Analyses of the different genes on nucleotide and amino acid level revealed that the non-structural ORF1a/1b are more conserved among α-CoVs and β-CoVs, while there are higher variations in the structural proteins known to be important for host specificity. The novel strain was named batCoV/MinFul/2018/SriLanka and had a prevalence of 50% (66/130) in rectal swab samples and 58% (61/104) in feces samples that were collected from Miniopterus bats in Wavul Galge cave. Based on the differences between strain batCoV/MinFul/2018/SriLanka and human-pathogenic α-CoVs and β-CoVs, we conclude that there is a rather low transmission risk to humans. Further studies in the Wavul Galge cave and at other locations in Sri Lanka will give more detailed information about the prevalence of this virus.

Keywords: bat alphacoronavirus; *Miniopterus fuliginosus*; Sri Lanka; cave-dwelling; sympatric colony; full genome; coronavirus; one health

1. Introduction

Coronaviruses (CoVs) are the members of the family Coronavirinae within the order Nidovirales, and they can cause respiratory diseases in animals and humans [1]. CoVs have

positive-sense, single-stranded RNA genomes with sizes between 27,000 and 32,000 nucleotides (nt) [2]. In general, the genome is organized in five major open reading frames (ORF), encoding a number of non-structural proteins for viral replication (ORF1a/1b), and the genes for the structural proteins of spike (S), membrane (M), envelope (E) and nucleocapsid (N) protein [3]. While the ORF1a/1b-coding sequences (CDS) are generally considered as highly conserved gene sections since they need to maintain protein functionality, the structural proteins are rather susceptible to substitutions on gene and protein levels. These variations, especially in the spike protein, can lead to differences in their infectivity and host specificity. In general, CoVs can be divided into the genera of α-CoVs, β-CoVs, γ-CoVs and δ-CoVs [4]. While γ-CoVs and δ-CoVs are probably derived from bird CoVs, α-CoVs and β-CoVs can be found in a variety of bat species, which are being discussed as their potential natural reservoir [5]. These two genera are also prevalent in a wide range of other mammals (wildlife and domestic animals) and humans. Viruses of the genus β-CoV such as SARS-CoV, MERS-CoV and SARS-CoV-2 can cause severe respiratory symptoms in humans, and their easy spread among humans holds the proven risk of pandemic developments. With the latest emergence of SARS-CoV-2 there are seven known CoVs that are capable of infecting humans [6]. α-CoVs are also capable of causing infections in humans; HCoV-229E and HCoV-NL63 cause mild upper respiratory diseases in humans and appear seasonally [7]. A thorough genomic characterization and understanding of the genotypical and phenotypical differences between α-CoVs and β-CoVs can help to provide understanding of the human-pathogenic potential of different CoVs.

In Sri Lanka, a high biodiversity in general and in particular a high bat species variety can be observed [8]. Although some of the 30 different bat species in Sri Lanka are described to be a potential reservoir for CoVs in other countries, there is only little knowledge on the prevalence of chiroptera-hosted CoVs on the Sri Lankan island so far [9]. In this study, a population of different bat species roosting in one of the largest natural caves (Wavul Galge, Koslanda, Sri Lanka) was examined. In a previous study, we have reported the detection of novel α-CoV fragments in feces and rectal swabs from a number of *M. fuliginosus* bats [10]. In this study, we present the first full genome of an α-CoV detected in Sri Lankan bats.

2. Materials and Methods

This study was carried out according to the relevant guidelines and regulations of the Fauna and Flora Protection Ordinance, approved by the local government authority (Department of Wildlife Conservation, Sri Lanka, permit No. WL/3/2/05/18, issued on 10 January 2018).

2.1. Bat Sampling

Sampling of cave-dwelling bats roosting in the Wavul Galge cave (Sri Lanka) was performed in March and July 2018 and January 2019 as described before [10]. Adequate personal protective equipment such as gloves, safety glasses and FFP3 masks were worn during the capturing and sampling procedure. All lab procedures were conducted under Biosafety level-2 conditions with appropriate precautions.

Bats were captured by using hand nets when leaving the cave at nightfall and were kept in bat holding bags until further processing. Bat species were determined by using macroscopic identifiers and documented together with other features such as weight, forearm length, sex and stage of age. For molecular species identification based on the cytochrome B gene, an oral swab was taken from each bat. Furthermore, fresh fecal pellets were collected with forceps from the holding bags if available, or else a rectal swab was taken. Samples were snap-frozen by using liquid nitrogen before storage at −80 °C until further processing.

In all three sampling sessions (March and July 2018, January 2019) a total of 395 bats were sampled, all belonging to the genera Miniopterus, Rousettus, Hipposideros and Rhinolophus (based on macroscopic species identification).

2.2. Shotgun NGS

For processing of rectal swabs, 500 µL of sterile PBS were added and mixed by vortexing. After a centrifugation step, 140 µL of the supernatant was used for extraction with a viral RNA mini kit (QIAGEN, Hilden, Germany).

To obtain the full genome of a bat α-CoV, 65 rectal swabs from sampling session July 2018 (only *M. fuliginosus*) were prepared for shotgun NGS. Before further processing, 5 to 10 RNA samples were pooled. Pools were digested at 37 °C for 30 min by using the TURBO DNA-free Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. For cDNA synthesis, SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers were used. The cDNA was used for second strand synthesis by using the NEBNext® Ultra™ II Non-Directional RNA Second Strand Synthesis Module (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. dsDNA was purified by using the Agencourt AMPure XP bead system (Beckman Coulter Life Sciences, Krefeld, Germany), adding 120 µL of magnetic beads per sample for binding, followed by two washing steps with 200 µL of 70% ethanol and an elution in 40 µL of PCR grade water. DNA concentration was determined by using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Hennigsdorf, Germany). Samples were sequenced on a HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) with a paired end read output of 2 × 250 bp and a total output of up to 7.5 million reads per pool.

2.3. NGS Data Analysis and Full Genome Assembly

Trimmed data were analyzed by using a diamond tool [11] and BLASTx algorithm with the "- - sensitive" setting. BLAST results were visualized in MEGAN [12] and Geneious Prime software (version 2020.2.3, Biomatters Ltd., Auckland, New Zealand).

In order to obtain the full genome sequence of the novel bat coronavirus, reads of all rectal swab pools were mapped to a reference sequence (BtMf-AlphaCoV/AH2011—Accession No. KJ473795) by using Geneious Prime software. The consensus sequence was calculated from the final assembly by using Geneious Prime software with distinct quality settings.

The quality of the generated consensus sequence was further validated. For areas with lower coverage or gaps in the consensus sequence, spanning primer pairs were designed based on the sequence data already available. RNA of the single rectal swab samples before pooling was transcribed to cDNA as described before and amplified with the respective spanning primers by using a standard PCR protocol (available upon request) and the Taq DNA Polymerase Kit (Invitrogen, Carlsbad, CA, USA). PCR products were analyzed by using agarose gel electrophoresis. Positive PCR products with distinct bands visible in the agarose gel were Sanger sequenced by using the BigDye Terminator Cycle Sequencing Kit on an Applied Biosystems 3500 Dx Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) with the respective forward and reverse primers.

PCR products with multiple bands were purified by using the MSB Spin PCRapace Kit (Invitrogen, Carlsbad, CA, USA) and sequenced by using MinION sequencing (Oxford Nanopore Technologies, Oxford, UK). The sequences were analyzed by using Geneious Prime software and mapped to the already existing consensus sequence as described before. After obtaining a full genome sequence, genes were annotated by using the annotation tool in the Geneious Prime software and Glimmer annotation tool [13–15].

2.4. Sequence Analysis and Phylogenetic Reconstruction

For phylogenetic reconstruction, the full genome as well as ORF1b CDS were used, respectively. A number of reference sequences were selected as representatives for the different subgenera of α-CoVs and β-CoVs and were downloaded from the NCBI database (Table 1). Recombination analysis of the full genome sequence and two closely related strains was performed using DualBrother detection software [16,17]. For phylogenetic reconstruction, nucleotide alignment of all selected representative CoV strains was calculated by using the MAFFT algorithm [18]. Phylogenetic trees were calculated by using MrBayes

version 3.2.6 [19]. The model GTR with gamma-distributed rate variation was selected for the calculations, and the parameters were set as follows: number of runs: four; number of generations: 500,000 to 1,000,000; subsampling frequency: 100 and burn-in: 10%. The phylogenetic trees were visualized using Geneious prime software.

Table 1. Overview of α-Coronaviruses (-CoVs), β-CoVs and γ-CoV from the NCBI database that were selected for the genomic and phylogenetic analyses.

Genus	Subgenus	Accession No.	Description
α-CoV	Minunacovirus	KJ473795	BtMf-AlphaCoV/AH2011
		NC_010437	Bat coronavirus 1A
		EU420137	Bat coronavirus 1B strain AFCD307
		EU420138	Miniopterus bat coronavirus 1
		EU420139	Bat coronavirus HKU8 strain AFCD77
		KJ473797	BtMf-AlphaCoV/GD2012
		NC_010438	Miniopterus bat coronavirus HKU8
	Pedacovirus	NC_009657	Scotophilus bat coronavirus 512
		MK211372	BtSk-AlphaCoV/GX2018D
	Decacovirus	MK720945	Rhinolophus bat coronavirus HKU32 strain TLC26A
		MN611523	Hipposideros pomona bat coronavirus HKU10-related isolate 160942
		NC_018871	Rousettus bat coronavirus HKU10
	Duvinacovirus	NC_002645	Human coronavirus 229E
		KY073747	229E-related bat coronavirus strain BtKY229E-1
	Setracovirus	NC_005831	Human coronavirus NL63
		NC_048216	NL63-related bat coronavirus strain BtKYNL63-9b
	Rhinacovirus	NC_009988	Rhinolophus bat coronavirus HKU2
		NC_028824	BtRf-AlphaCoV/YN2012
	Myotacovirus	NC_028811	BtMr-AlphaCoV/SAX2011
		KY770851	Bat coronavirus isolate Anlong-57
	Colacovirus	NC_022103	Bat coronavirus CDPHE15/USA/2006
		KY799179	Myotis lucifugus coronavirus
	Nyctacovirus	NC_028833	BtNv-AlphaCoV/SC2013
		NC_046964	Alphacoronavirus bat-CoV/P.kuhlii/Italy/3398-19/2015
		MK720944	Tylonycteris bat coronavirus HKU33 strain GZ151867
β-CoV	Embecovirus	AY391777	Human coronavirus OC43
		NC_006577	Human coronavirus HKU1
	Sarbecovirus	NC_004718	SARS coronavirus Tor2
		NC_045512	SARS coronavirus 2 isolate Wuhan-Hu-1
		MN996532	Bat coronavirus RaTG13
		DQ022305	Bat SARS coronavirus HKU3-1
	Hibecovirus	NC_025217	Bat Hp-betacoronavirus/Zhejiang2013
	Merbecovirus	NC_009019	Tylonycteris bat coronavirus HKU4
		NC_009020	Pipistrellus bat coronavirus HKU5
		KX442565	Hypsugo bat coronavirus HKU25 isolate NL140462
		NC_019843	MERS-related coronavirus isolate HCoV-EMC/2012
	Nobecovirus	NC_009021	Rousettus bat coronavirus HKU9
		MT350598	Rousettus bat coronavirus GCCDC1
γ-CoV	Igacovirus	NC_001451	Avian infectious bronchitis virus

2.5. Real-Time RT-PCR Design

To determine the prevalence of the novel α-CoV in the examined bat population, a specific real-time RT-PCR was designed based on the obtained sequencing data. A region on

the conserved ORF1b CDS (nt position 16,286–16,406) with high read coverage was selected, and a forward primer (TggTTTTTgTgTTAACATCACAT), a reverse primer (gCAAAAT-CACTACTAATATTgAACAC) and a probe (FAM-ACCACCTTTgAgAgCTCCTACAATCgC-BHQ1) were designed, producing a 121 bp amplicon (Figure 1). The specificity of the assay was tested in silico by mapping the primers to the reference strains (Table 1), allowing up to 5 mismatches. In addition, the specificity of the PCR assay was tested in vitro with other available samples of α-CoVs and β-CoVs. For quantification of the samples, in vitro RNA (ivRNA) of the 121 bp amplicon was synthesized (GenExpress, Berlin, Germany). Using the ivRNA, the sensitivity of the assay was validated. The detection limit was 10 copies per reaction. The final PCR protocol involved the AgPath ID Kit (Invitrogen, Carlsbad, CA, USA) with 3 µL of RNA sample. The thermal profile included a reverse transcription step at 45 °C for 15 min, followed by 90 °C for 10 min. PCR cycling was performed at 95 °C for 15 s and 60 °C for 30 s for overall 45 cycles. The ivRNA was measured in different concentrations parallel to the samples to calculate the viral load per sample.

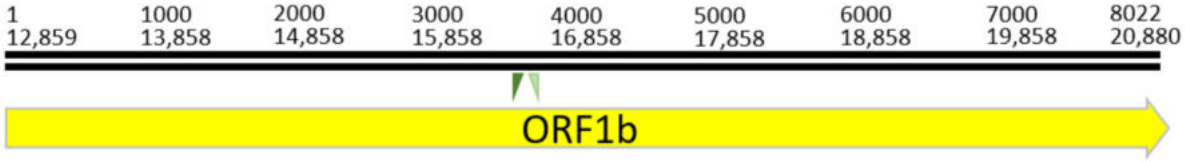

Figure 1. Location of the newly designed batCoV real-time RT-PCR assay (green labels) on the ORF1b gene (yellow), producing an amplicon of 121 bp at nucleotide position 16,286–16,406.

RNAs of all rectal swab and feces pellets, collected at the three sampling points, were tested with the newly designed bat α-CoV real-time RT-PCR assay to determine the prevalence of the new strain in the samples set.

3. Results

In this study, nine pools containing a total of 65 rectal swab samples were sequenced and subsequently analyzed. All these samples were collected from *M. fuliginosus* bats during the sampling session in July 2018.

3.1. NGS, Full Genome Assembly and Gene Organization

From NGS data, a total of 7332 reads were mapped to the BtMf-AlphaCoV/AH2011 reference genome (KJ473795) that covered almost the whole genome. Fifteen gaps or low-quality areas were additionally sequenced by using the Sanger and MinION sequencing method as described before. Finally, the complete genome of the novel bat coronavirus with a length of 27,987 nucleotides was obtained. The novel strain was named batCoV/MinFul/2018/SriLanka according to the literature. The full genome sequence of the batCoV/MinFul/2018/SriLanka is available at GenBank (OL956935).

The analysis of the full genome revealed a similar gene organization as the reference genome BtMf-AlphaCoV/AH2011 and other α-CoVs from Miniopterus bats, starting with the CDS of the non-structural polyproteins ORF1a/1b, followed by the genes for the structural spike protein, envelope, membrane and nucleocapsid protein (Table 2). All CoVs are known to have a universal frame shift in the ORF1a/1b polyprotein with the sequence pattern U_UUA_AAC [20,21]. With this, a ribosomal frameshift is induced and allows the translation of the ORF1b polyprotein. The polyprotein ORF1a/1b of the novel strain batCoV/MinFul/2018/SriLanka was annotated accordingly.

In order to classify the genome of batCoV/MinFul/2018/SriLanka, comparative analysis of the complete genome and genes was performed with selected reference strains of α-CoVs and β-CoVs. Table 3 gives an overview on the pairwise identity of the novel batCoV/MinFul/2018/SriLanka with selected references on nucleotide (nt) and amino acid (aa) level.

Table 2. Annotated coding sequences (CDS) of the novel batCoV/MinFul/2018/SriLanka strain compared to the reference genome KJ473795 (BtMf-AlphaCoV/AH2011).

	CDS	Start–End (Nucleotide Position)	No. of Nucleotides	No. of Amino Acids
batCoV/MinFul/2018/SriLanka	ORF1a	113–12,859	12,747	4249
	ORF1b	12,859–20,880	8022	2674
	Spike	20,882–25,009	4128	1376
	Envelope	25,662–25,886	225	75
	Membrane	25,893–26,651	759	253
	Nucleocapsid	26,672–27,841	1170	390
KJ473795	ORF1a	273–13,046	12,774	4258
	ORF1b	13,046–21,067	8022	2674
	Spike	21,069–25,196	4128	1376
	Envelope	25,849–26,073	225	75
	Membrane	26,080–26,841	762	251
	Nucleocapsid	26,862–28,031	1170	390

Table 3. Pairwise nucleotide (nt) and amino acid (aa) identities of the novel batCoV/MinFul/2018/SriLanka strain compared to selected α-CoVs and β-CoVs.

	Pairwise Nucleotide Identity (%)							Pairwise Amino Acid Identity (%)					
	Full Genome	ORF1a	ORF1b	S	E	M	N	ORF1a	ORF1b	S	E	M	N
Alphacoronaviruses													
Miniopterus bat coronavirus 1	84.9	83.0	89.2	83.1	92.4	88.1	88.5	86.1	94.9	87.1	91.9	88.7	89.5
BtMf-AlphaCoV/AH2011	85.1	83.2	89.7	83.2	92.9	86.5	89.2	86.6	95.5	87.7	91.9	87.5	98.7
BtMf-AlphaCoV/GD2012	66.8	65.5	77.7	62.5	70.7	73.9	58.0	63.3	87.4	59.1	67.6	74.5	57.2
Human coronavirus 229E	57.0	54.9	71.7	46.7	57.6	54.3	43.3	47.7	77.5	45.0	44.7	56.2	37.8
Human coronavirus NL63	59.4	55.7	73.3	52.8	61.5	56.0	49.3	47.7	77.6	43.5	51.3	60.6	45.5
Betacoronaviruses													
Bat SARS coronavirus HKU3-1	39.4	34.3	58.0	32.6	34.6	39.3	32.2	19.7	56.1	18.1	21.3	30.5	22.4
Rousettus bat coronavirus HKU9	39.1	33.2	57.9	34.0	41.5	43.6	30.6	19.7	55.8	18.3	15.6	31.6	18.5
Pipistrellus bat coronavirus HKU5	38.0	32.4	57.1	34.2	37.4	38.4	30.8	20.5	56.2	19.0	16.3	32.6	24.1
Human coronavirus HKU1	42.8	39.9	58.5	38.6	42.8	42.6	33.5	20.3	53.3	18.2	17.7	34.6	23.7
SARS-CoV-2 Wuhan-Hu-1	40.2	34.7	59.6	34.4	35.5	39.6	31.9	20.2	56.5	18.3	20.0	30.8	21.7
Human coronavirus OC43	40.3	39.0	57.3	37.7	40.3	41.1	32.0	20.3	53.2	18.1	17.9	32.5	21.0

ORF: open reading frame; S: spike CDS; E: envelope CDS; M: membrane CDS; N: nucleocapsid CDS.

BatCoV/MinFul/2018/SriLanka shares the highest identities with other α-CoVs from Miniopterus bats; identities among all α-CoVs used in this analysis ranged from 57.0 to 85.1% on the nucleotide level of the full genome comparative analysis. In contrast, the novel batCoV/MinFul/2018/SriLanka shared identities of 38–42.8% with the selected β-CoVs.

3.1.1. Non-Structural Protein CDS: ORF1a/1b

Based on the alignments of the non-structural polyprotein CDS ORF1a/1b (Table 3), the novel batCoV/MinFul/2018/SriLanka shared the highest identities with the Miniopterus bat coronavirus 1 and the strain BtMf-AlphaCoV/AH2011. For ORF1a, calculated nucleotide identities were ≥83% (aa level: ≥86.1%). In contrast, the human-related α-CoVs 229E and NL63 showed lower nucleotide identities of around 55% (aa: 47.7%). All β-CoVs showed lower nucleotide identities of 32.4–39.9% (aa: 19.7–20.5%) compared to the α-CoVs. In general, the ORF1b segment of the non-structural ORF1a/1b polyprotein showed higher identities compared to ORF1a. Especially the comparative analysis to the β-CoVs showed identities of 57.1–59.6% on nucleotide level (aa: 53.2–56.5%), which is remarkably higher than the identities calculated for the complete genomes and for the other structural proteins.

3.1.2. Structural Protein CDS: Spike, Envelope, Membrane, Nucleotide

The comparative analysis of the structural protein CDS generally reflected the results of the analysis of the full genomes. Despite this, the spike protein of all β-CoVs showed ~5% lower identities compared to the pairwise identities of the full genome. The same applies to the β-CoV sequences of the envelope and nucleocapsid proteins, while the membrane proteins showed higher identities comparable to the pairwise identities of the full genomes.

Interestingly, the membrane proteins of the novel batCoV/MinFul/2018/SriLanka and other α-CoVs from the subgenus Minunacovirus have lengths of 253–256 aa, while the remaining α-CoVs and β-CoVs have lengths of 219–236 aa. These additional amino acids in the Minunacovirus sequences are located at the beginning of the membrane protein sequence (Figure 2).

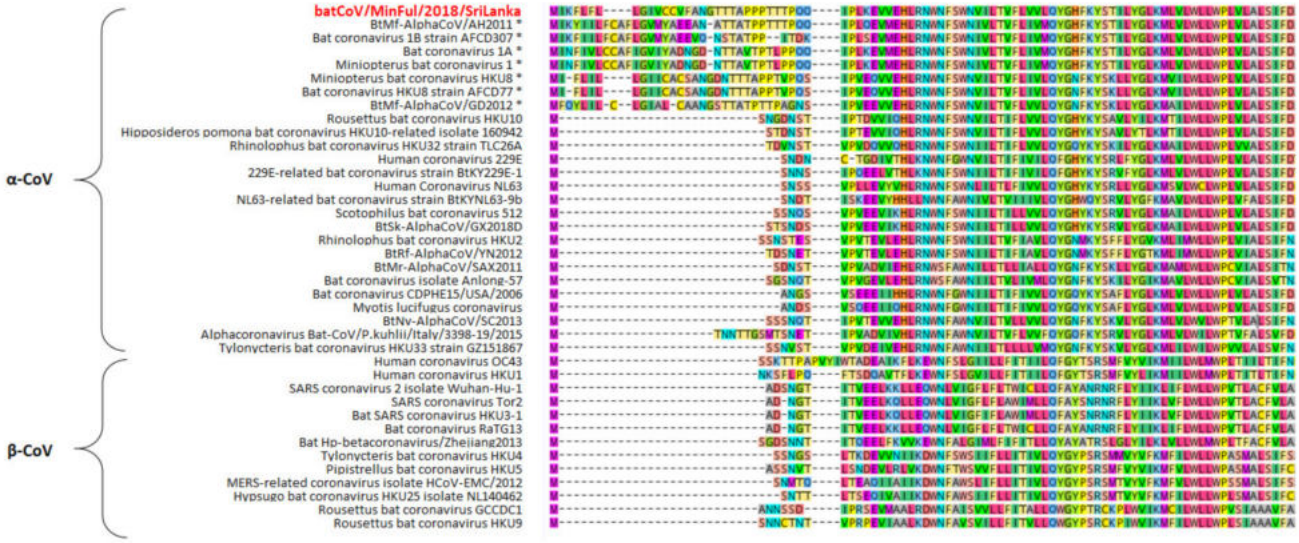

Figure 2. Extract of the first 100 amino acids (aa) of a multiple sequence alignment of the membrane protein from different α-CoVs and β-CoVs, calculated with MAFFT algorithm and visualized in Geneious Prime software with a color code for each aa. The novel strain batCoV/MinFul/2018/SriLanka is marked in red. α-CoVs of the subgenus Minunacovirus are marked with an asterisk.

For the spike protein sequences, the multiple alignments showed high variation in the first section (aa 1–596 of the batCoV/MinFul/2018/SriLanka spike CDS) with identities of 10% and less between α-CoV and β-CoV strains. The second section (aa 597–1375 of the batCoV/MinFul/2018/SriLanka spike CDS) showed higher similarities between α-CoV and β-CoV, ranging from 24.9 to 28.9%.

To sum up, the sequence differences between α-CoVs and β-CoVs of different subgenera were visualized with this comparative analysis. In general, we found highly conserved

regions in the second part of the ORF1a CDS and the complete ORF1b CDS, which shared high identities among all analyzed α-CoV and β-CoV strains. The remaining parts of the ORF1a CDS and all the structural protein CDS showed higher diversity between α-CoVs and β-CoVs and also among each other.

3.2. Phylogeny

A phylogenetic tree (Figure 3) was calculated for the full genome based on a nucleotide alignment with all selected reference sequences of the α-CoV and β-CoV subgenera. The calculation reveals the separation of these strains into distinct clades, α-CoVs and β-CoVs. In the α-CoV clade, strain batCoV/MinFul/2018/SriLanka clusters with other α-CoVs of the subgenus Minunacovirus. These are the strains that shared the highest overall pairwise identities with the novel batCoV/MinFul/2018/SriLanka strain on nt and aa level.

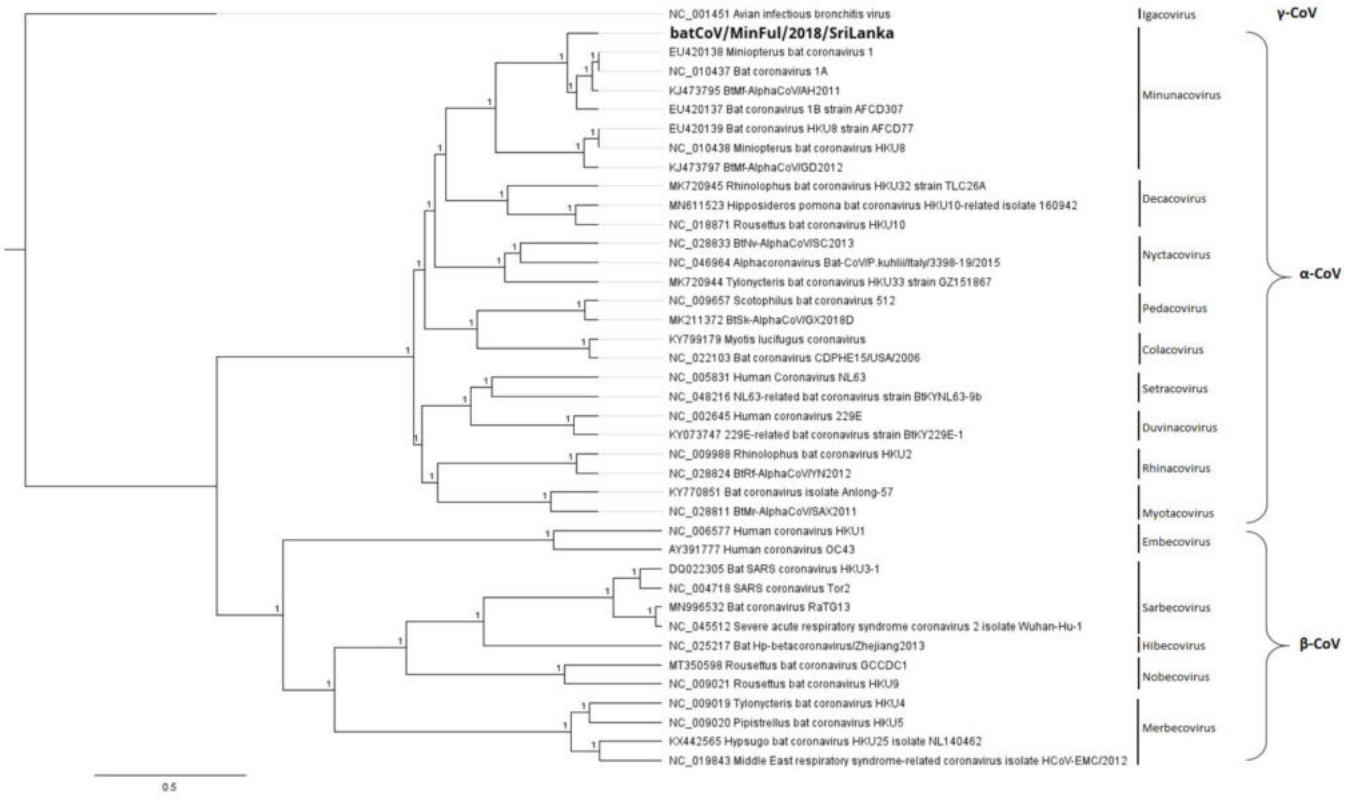

Figure 3. Phylogenetic tree based on a full genome nucleotide (nt) alignment of the novel strain batCoV/MinFul/2018/SriLanka (bold) with selected α-CoVs and β-CoVs and specification of the subgenera. The γ-CoV avian infectious bronchitis virus (NC_001451) was included as an outgroup for the calculation. The phylogenetic tree was calculated with Bayesian algorithm, and 500,000 generations were calculated with a subsampling frequency of 100 and a burn-in of 10%. Substitution model GTR was selected with a gamma-distributed rate variation.

The remaining α-CoV strains form separate branches according to the assigned subgenera. For the β-CoV branch, the separation into the subgenera is clearly represented (compare Figure 3).

In addition to the phylogenetic reconstruction of the full genomes, the conserved ORF1b CDS was selected for phylogenetic analysis with further CoV strains. The phylogenetic tree (Figure 4) is based on a gap-free nucleotide alignment of the representative CoV strains of different subgenera, and the associated heatmap (Figure 5) displays the distances among these strains on an amino acid level.

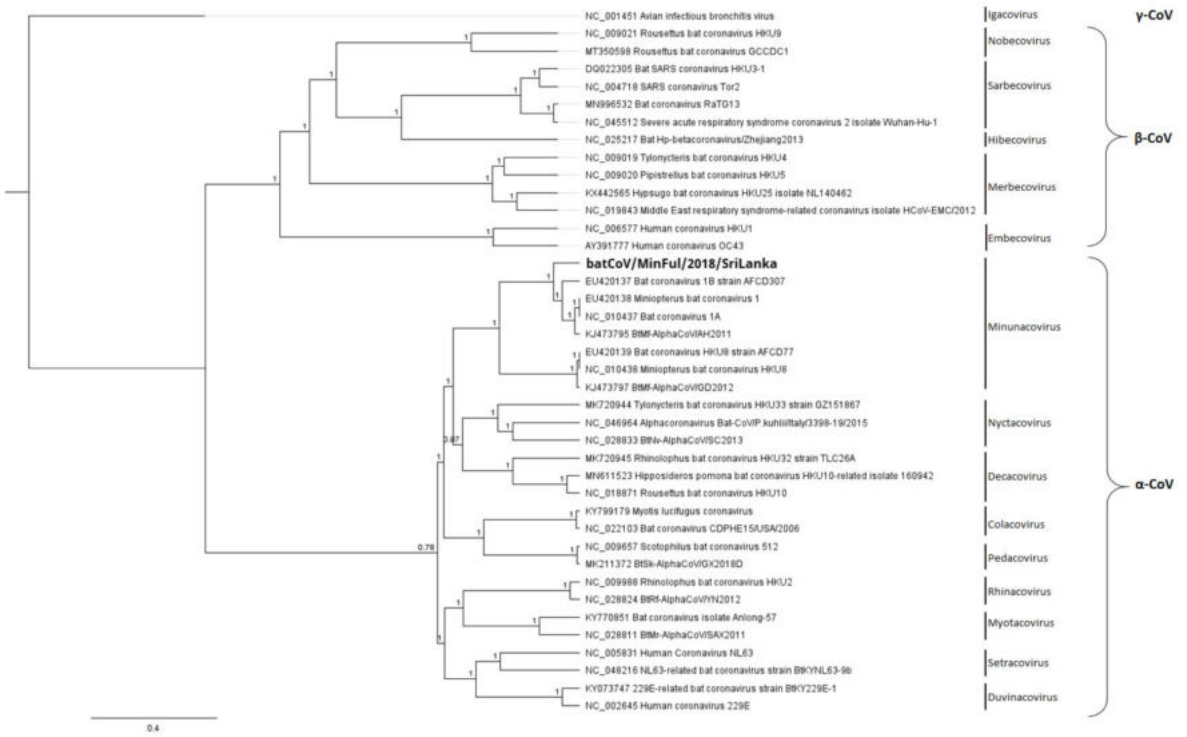

Figure 4. Phylogenetic tree based on an ORF1b nt alignment of the novel bat-CoV/MinFul/2018/SriLanka (bold) and selected CoV strains from different subgenera. The γ-CoV avian infectious bronchitis virus (NC_001451) was included as an outgroup for the calculation. The phylogenetic tree was calculated with the Bayesian algorithm, and 1 million generations were calculated with a subsampling frequency of 100 and a burn-in of 10%. Substitution model GTR was selected with a gamma-distributed rate variation.

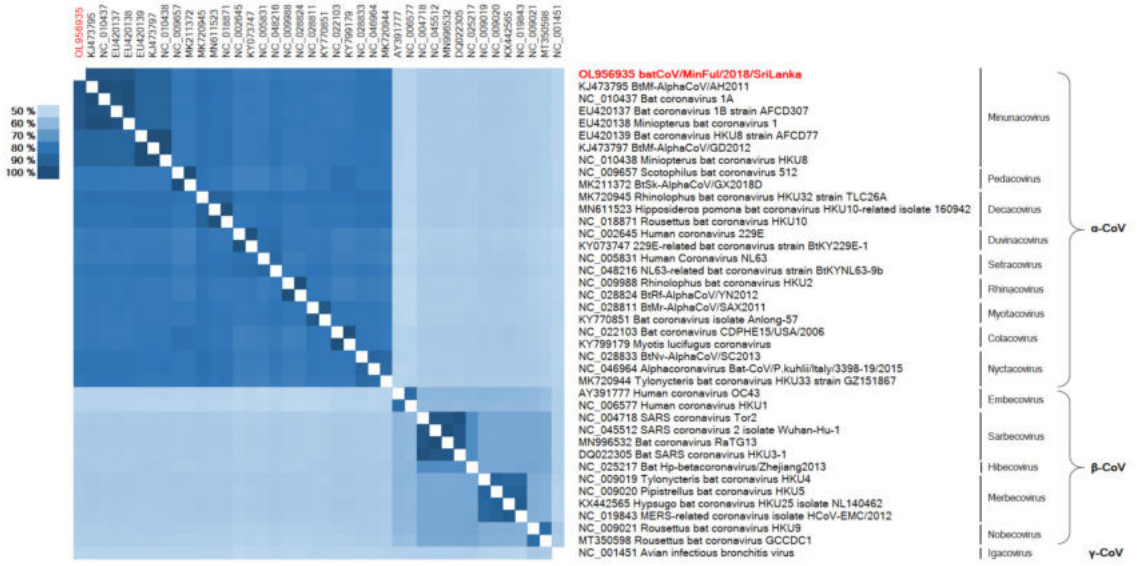

Figure 5. Heatmap based on an ORF1b aa alignment of the novel batCoV/MinFul/2018/SriLanka (red) and 39 selected CoV strains.

In Figure 4 the phylogenetic reconstruction of batCoV/MinFul/2018/SriLanka ORF1b along with representative α-CoVs and β-CoVs is displayed. The γ-CoV strain NC_001451 (avian infectious bronchitis virus) was selected as the outgroup and used to root the phylogenetic tree. Within the β-CoVs clade, the separate subclades are visible, representing the

subgenera Hibecovirus, Embecovirus, Sarbecovirus, Nobecovirus and Merbecovirus. These clusters are clearly visible in the associated heatmap as well (Figure 5). Within the α-CoV clade, the tree is partitioned into several subclades representing the subgenera, respectively. Inside the subclade of Minunacoviruses, the novel batCoV/MinFul/2018/SriLanka strain is clustered with α-CoV strains of the Miniopterus bat coronavirus 1 species. In this Minunacovirus subclade, the Miniopterus bat coronavirus HKU8-related strains build a separate branch. The distances between Miniopterus bat coronavirus 1 strains and HKU8 strains were confirmed with the associated heatmap as well (Figure 5).

3.3. Validation of NGS Results Using Real-Time RT-PCR

In order to validate the results of NGS data from the nine pool samples and test them back in the individual rectal swab samples, a specific real-time RT-PCR was designed. Specificity of the assay was tested with a number of other α-CoVs (HCoV-229E, -NL63) and β-CoVs (SARS-CoV, MERS-CoV, HCoVs-OC43). As no unspecific amplification was observed, a high specificity of the PCR assay to the novel batCoV/MinFul/2018/SriLanka can be assumed. All individual 65 rectal swabs from the analyzed NGS pools were tested with the newly designed real-time RT-PCR assay, and positive results were quantified by using a standardized synthetic RNA control (Table 4).

Table 4. Overview of positive results after screening of the rectal swab with the newly designed real-time RT-PCR assay for the detection of the novel batCoV/MinFul/2018/SriLanka; copy numbers of 15 and below are shown in brackets.

Pool	Positive Samples/Total	Positive Sample	Sampling Date	Sex	Copies per Reaction (25 µL)
RS 2.2	6/8	RS 85	7 July 2018	M	(1)
		RS 91	7 July 2018	F	(14)
		RS 94	7 July 2018	M	(12)
		RS 95	7 July 2018	F	1313
		RS 96	7 July 2018	F	60
		RS 98	7 July 2018	F	468
RS 2.3	4/8	RS 106	7 July 2018	F	106
		RS 108	7 July 2018	F	371
		RS 109	7 July 2018	M	854
		RS 110	7 July 2018	F	1737
RS 2.4	4/7	RS 114	7 July 2018	F	70
		RS 117	7 July 2018	F	46
		RS 118	7 July 2018	F	(14)
		RS 119	7 July 2018	F	650
RS 2.5	3/7	RS 124	7 July 2018	F	105
		RS 126	7 July 2018	F	1391
		RS 133	7 July 2018	M	242
RS 2.6	3/6	RS 135	7 July 2018	F	339
		RS 137	7 July 2018	F	2124
		RS 138	7 July 2018	F	106
RS 2.7	2/7	RS 146	7 July 2018	F	44
		RS 147	7 July 2018	F	93

Table 4. *Cont.*

Pool	Positive Samples/Total	Positive Sample	Sampling Date	Sex	Copies per Reaction (25 µL)
RS 2.8	5/9	RS 154	8 July 2018	F	2611
		RS 158	8 July 2018	F	71
		RS 159	8 July 2018	F	1196
		RS 162	8 July 2018	F	866
		RS 164	8 July 2018	F	33
RS 2.9	7/8	RS 168	8 July 2018	F	296
		RS 169	8 July 2018	F	76
		RS 170	8 July 2018	F	557
		RS 171	8 July 2018	M	196
		RS 172	8 July 2018	F	104
		RS 175	8 July 2018	F	464
		RS 176	8 July 2018	F	753
RS 2.10	2/5	RS 178	8 July 2018	F	600
		RS 187	8 July 2018	F	(5)

Overall, 36 out of the 65 rectal swabs (55%) tested positive for batCoV/MinFul/2018/SriLanka with viral loads of up to 2611 copies per reaction. The sensitivity of the newly designed real-time RT-PCR assay was 10 copies per reaction; therefore, all PCR-positive samples with concentrations of less than 15 copies per reaction were considered as uncertain positive results.

Of the 65 bats, the majority was female. To put this into context, of all 200 sampled bats that were sampled during the session in July 2018, a high number of 171 bats (85%) were females and only 29 captured bats were males.

In order to gain an overall prevalence of batCoV/MinFul/2018/SriLanka, we additionally tested all collected rectal swab and feces samples from all species and all time points with the specifically designed real-time RT-PCR (Table 5).

Table 5. Overview of the rectal swab and feces samples and pools per bat genus, collected at three different time points. Only samples from Miniopterus bats tested positive for the novel batCoV/MinFul/2018/SriLanka.

Genus	Miniopterus		Rousettus		Hipposideros		Rhinolophus	
	Rectal Swabs	Feces	Rectal Swabs	Feces	Rectal Swabs	Feces	Rectal Swabs	Feces
March 2018								
Pools	2	0	1	1	1	0	12	1
Positive samples	3/3	0/0	0/9	0/2	0/3	0/0	0/60	0/8
July 2018								
Pools	17	8	1	0	0	0	0	0
Positive samples	59/116	38/77	0/11	0/0	0/0	0/0	0/0	0/0
January 2019								
Pools	1	3	2	1	3	2	2	2
Positive samples	4/11	23/27	0/16	0/3	0/16	0/7	0/16	0/17
Total positive	66/130 (50%)	61/104 (58%)	0/36	0/5	0/19	0/7	0/76	0/25

4. Discussion

In our study, we were able to obtain the full genome of an α-CoV strain named batCoV/MinFul/2018/SriLanka from *Miniopterus fuliginosus* bats from Sri Lanka. With our comparative analysis on the full genome level, we found overall high identities to the Miniopterus bat coronavirus 1, which belongs to the subgenus of Minunacoviruses within the α-CoVs. Therefore, we suggest that the detected batCoV/MinFul/2018/SriLanka belongs to the same subgenus. According to ICTV criteria, the demarcation threshold of a novel virus species within the coronavirus family is an aa identity of less than 90% in the conserved replicase domains [22,23]. With an identity of 95.5% to the reference strain BtMf-AlphaCoV/AH2011 (KJ473795), we therefore assume that the Sri Lankan strain belongs to the same virus species Miniopterus bat coronavirus 1.

The recombination analysis did not show signs of recombination between the novel batCoV/MinFul/2018/SriLanka and other strains of the Minunacovirus subgenus. The genetic comparison also showed that the novel batCoV/MinFul/2018/SriLanka has rather low identities to the human-pathogenic strains HCoVs 229E and NL63 compared to the other bat-related α-CoVs. For both HCoVs, different most common ancestors are assumed, namely a Hipposideros bat species for HCoV 229E and a Perimyotis bat species for HCoV NL63 [24].

In our study, we used the specifically designed rt-PCR to screen for the novel batCoV/MinFul/2018/SriLanka strain in all rectal swab and feces samples collected from Miniopterus, Hipposideros, Rhinolophus and Rousettus bats. Although all bats live in a sympatric colony in the Wavul Galge cave, the virus was detected only in *M. fuliginosus* bats. This supports the assumption that the batCoV/MinFul/2018/SriLanka strain has a high host specificity to Miniopterus bats; this also seems reasonable because of its high resemblance to Miniopterus-related viruses and lower similarity to other α-CoVs. This assumption is supported by the phylogenetic analysis where the branch of HCoVs is separated early from the remaining α-CoVs.

The high overall divergence between α-CoVs and β-CoVs was also demonstrated by means of the comparative and phylogenetic analysis. Again, this may be explained by the different natural hosts of α-CoVs and β-CoVs. Miniopterus bats and other species of the family Vespertilionidae are mainly known to carry α-CoVs, while β-CoVs are rather found in other Chiroptera families [25]. SARS-related β-CoVs are mainly found globally in Rhinolophus bats but were also detected in Hipposideros and Chaerephon bat species in Africa [25]. We reveal that the high diversity between the chiroptera-hosted CoVs may be explained by a host-related evolution of the viruses and is driven by geographically distinct bat species, which is in line with a prior study [26]. The phylogenetic trees likewise show an early separation of the α-CoV and β-CoV clades.

The main differences between the novel batCoV/MinFul/2018/SriLanka strain and other α-CoVs and β-CoVs are found in the structural proteins. Especially the amino acid sequences of spike, envelope and nucleocapsid showed the highest diversity among all strains. The spike protein is responsible for the virus attachment to the host cell and the fusion with the cellular membrane [7]. Since this process is very host specific, a high diversity of this protein can be assumed. The spike protein consists of two subunits, S1 and S2. The S1 subunit is the peripheral part of the protein and responsible for recognizing and binding to the host ACE2 receptor [24,27]. In detail, the affinity of the specific receptor binding domain (RBD) to this ACE2 receptor affects the efficiency of viral binding and entry to the host cell [28]. The RBD of human pathogenic CoVs is different to that of other chiroptera-hosted CoVs. In addition, the presence of a furin cleavage site in the spike protein is known to enhance the cell entrance of SARS-CoV-2 and other human pathogenic CoVs to the human host cell [29]. Comparing the spike protein sequences of the novel batCoV/MinFul/2018/SriLanka strain to different human pathogenic CoVs, we could not find the same RBD sequence. Furthermore, the furin cleavage motif was not present in the batCoV/MinFul/2018/SriLanka strain. With these sequence differences, a rather low human pathogenic potential may be concluded.

The S2 subunit is an integral membrane part of the spike protein and responsible for the fusion of the virus particle with the host membrane [27]. This process is rather independent of the respective host cell; therefore, the S2 subunit is more conserved among different CoV strains. In our analysis we confirmed this, showing that the S1 subunit of the spike protein is highly diverse, while the S2 subunit showed higher identities between all compared CoV strains.

In contrast to the structural proteins, the ORF1a/1b CDS are generally higher conserved parts of the genome. The complete ORF1a/1b polyprotein forms the largest part of the CoV genome and codes for different non-structural proteins for replication [25]. The highest resemblance among all compared CoV strains was observed in the ORF1b CDS, especially in the first part of the polyprotein coding for the RNA-dependent RNA polymerase [30]. In contrast, the ORF1a sequences seem to be less conserved and are different in particular between α-CoVs and β-CoVs. It has been discussed that the ORF1a part is not only responsible for replication but also important for the survival of the virus and its adaptation to the respective host [30]. Our analysis would support this assumption, since the compared CoV strains with a high diversity in ORF1a CDS originate from diverse hosts, while strains with the same host generally share higher identities in the OFR1a CDS as well.

In conclusion, a high host specificity of the novel batCoV/MinFul/2018/SriLanka to Miniopterus bat species can be assumed, while a risk of transmission from bats to humans is estimated to be rather low. In order to investigate possible transmission to other species, we used the newly designed real-time RT-PCR not only for the verification of the batCoV/MinFul/2018/SriLanka in the previously analyzed Miniopterus samples, but also for screening of all rectal swabs and feces samples that were collected during the three sampling sessions in March 2018, July 2018 and January 2019. With this extended screening we found an additional number of positive samples in all three sessions. Interestingly, these samples were only from Miniopterus bats, although we sampled bats of the genus Hipposideros, Rhinolophus and Rousettus in all sampling sessions. All these bat species live in a sympatric colony in the Wavul Galge cave (Sri Lanka) where the sampling took place. Although the roosting sites for each species are separated inside the cave, the bats have contact when exiting and entering the cave. In addition, urine droppings inside the cave would facilitate a transmission route via aerosols. Nevertheless, we did not find any indications for the presence of the virus in other bat species so far. This emphasizes that the virus is host specific for *M. fuliginosus* and transmission to other species is not very likely. There are no indications that an α-CoV like the batCoV/MinFul/2018/SriLanka strain has a high potential to be transmitted to humans and to cause a pandemic comparable to that of SARS-CoV-2. Nevertheless, follow-up studies with a higher number of all bat species in the Wavul Galge cave, blood collection to check for seroconversion and sampling at different time points over the year could verify the assumption of host specificity and check for seasonal shedding of the virus in *M. fuliginosus*.

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Data Availability Statement: The data presented in this study are openly available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 31 January 2022), Accession number OL956935.

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Comparative virome analysis of individual shedding routes of Miniopterus fuliginosus *bats inhabiting the Wavul Galge Cave, Sri Lanka*

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Abstract

Bats are described as the natural reservoir host for a wide range of viruses. Although an increasing number of bat-associated, potentially human pathogenic viruses were discovered in the past, the full picture of the bat viromes is not explored yet. In this study, the virome composition from *Miniopterus fuliginosus* bats inhabiting the Wavul Galge cave, Sri Lanka, was analyzed. To assess different possible shedding routes, oral swabs, feces and urine were collected and analyzed individually by using metagenomic NGS. The data obtained was further evaluated by using phylogenetic reconstructions.

Two different alphacoronavirus strains were detected in feces and urine samples. Furthermore, a paramyxovirus was detected in urine samples. Sequences related to *Picornaviridae*, *Iflaviridae*, unclassified *Riboviria* and *Astroviridae* were identified in feces samples, and further sequences related to *Astroviridae* in urine samples. No further viruses were detected in oral swab samples.

The comparative virome analysis in this study revealed a diversity in the virome composition between the collected sample types which also represent different potential shedding routes for the detected viruses. At the same time, several viruses were detected for the first time in bats in Sri Lanka.

The detection of two different coronaviruses in the samples indicates the potential general persistence of this virus species in *M. fuliginosus* bats. Based on phylogenetics, the identified viruses are closer related to bat-associated viruses with comparably low human pathogenic potential. In further studies, the seasonal variation of the virome will be analyzed to identify possible shedding patterns for particular viruses.

Introduction

Bats are species-rich and taxonomically diverse mammals in the order *Chiroptera* that are distributed worldwide [1]. They represent a large group (20 %) of mammals and share unique features like their ability to fly. A number of viruses from different viral families, including human pathogenic viruses like Hendra and Nipah virus, coronaviruses, lyssaviruses and others have been associated with bats [2]. It is assumed that these viruses evolved together with their natural reservoir hosts. Because of this co-speciation and adaptation process, the viruses are often less pathogenic for their bat hosts. It is assumed that the bats' immune system is adapted

62 to and hence able to control virus infections without developing visible symptomatic diseases
63 [3–5].

64 With the increasing focus on bat virus research, the detection of potentially zoonotic viruses
65 with species-specific or family-specific PCR assays (e.g. paramyxoviruses, lyssaviruses,
66 coronaviruses) has been a convenient standard method. However, this may have led to a bias
67 focusing on certain viruses of particular interest [6–9]. To eliminate this disadvantage,
68 metagenomic NGS methods (mNGS) for virus discovery were applied allowing for an
69 untargeted and unbiased sequencing of novel viruses. The analysis of the whole virus
70 composition in bat samples (viromes) allows to reduce the bias and to constantly increase the
71 number of viral sequences deposited in sequence databases such as GenBank of the National
72 Center for Biotechnology Information (NCBI) [10–12].

73 However, in several regions of the world the investigation of bats in their role as potential
74 reservoir host of zoonotic viruses has been barely conducted [6]. In Sri Lanka, zoology is an
75 important research field and ecological aspects of bats are well investigated [13]. They
76 significantly contribute to the biodiversity and account for about a third of the Sri Lankan
77 mammals with 30 different species [14]. Furthermore, they are essential for the maintenance of
78 the ecosystem by providing ecoservices such as pollination, seed dispersal and insect control
79 [13].

80 In contrast, only few studies have focused on bats as reservoir host for pathogens in Sri Lanka
81 [15–18]. Here we present the first virome analysis of *M. fuliginosus* bats inhabiting Wavul
82 Galge cave (Koslanda, Sri Lanka) in the interior of Sri Lanka. *M. fuliginosus* bats are roosting
83 sympatrically with the other bat species *Hipposideros lankadiva, Hipposideros speoris,*
84 *Rhinolophus rouxii* and *Rousettus leschenaultii*. In three individual field studies at different
85 time points, we captured bats of all representative species and collected different sample
86 material depending on availability [19]. Selected sample sets had been analyzed in previous
87 investigations focusing on different research questions [17, 18, 20, 21]. In this study, we focus
88 on the virome analysis of urine swabs (US), oral swabs (OS) and feces (F) collected from
89 *M. fuliginosus* bats at one sampling point (July 2018). The presented results give insights into
90 the virome composition of this bat species in the Wavul Galge cave and in general. Furthermore,
91 the results reveal differences in viral shedding routes by analyzing the different sample types.

92

93

Methods

The study was approved by the local government authority (Department of Wildlife Conservation, Sri Lanka, permit No. WL/3/2/05/18, issued on 10 January 2018). Catching and sampling of bats was carried out according to relevant guidelines and regulations of the Fauna and Flora Protection Ordinance [19].

Bat Sampling

Sampling of bats inhabiting the Wavul Galge cave (Sri Lanka) was performed in March and July 2018 and January 2019 as described before [17]. For the results presented in this study, only bat samples from the species *M. fuliginosus* collected in July 2018 were included. 188 individuals of this bat species were captured and sampled with adequate personal protective equipment. Different sample types were collected depending on availability. From *M. fuliginosus* a total of 187 oral swabs (OS), 102 urine swabs (US) and 77 fecal pellets (F) were taken and analyzed.

The general workflow of subsequent laboratory work and bioinformatic analysis of NGS data is shown in Figure 1.

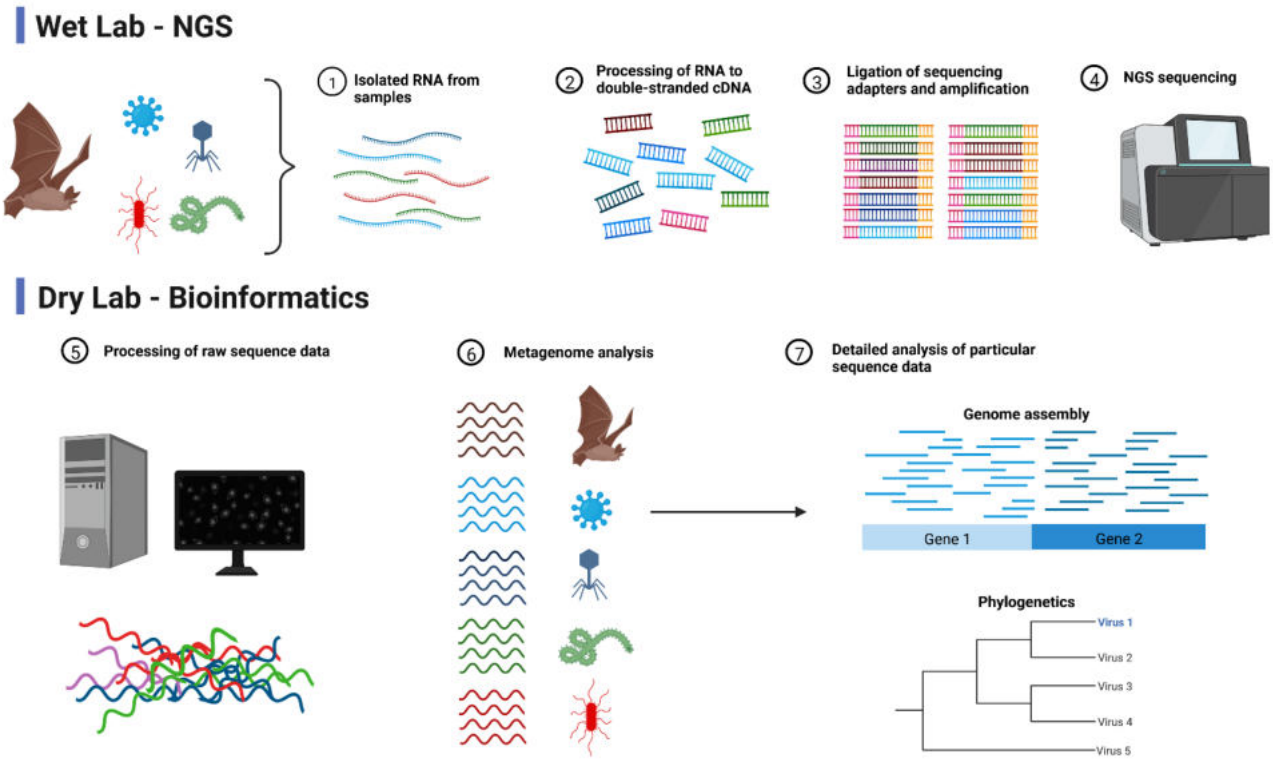

Figure 1: Schematic illustration of the general NGS workflow (wet lab) with subsequent bioinformatic analysis of obtained data (dry lab). The goal is to analyze the general virome composition (6) of the respective sample and to further characterize novel viruses based on the obtained sequencing data (7). Created with BioRender.com

Metagenomic NGS

The processing of samples was conducted with appropriate precautions in biosafety-2 laboratories. All samples were initially processed by adding 500 µL of sterile PBS. For oral swabs and urine swabs, samples were mixed by vortexing before 140 µL were used for extraction with the Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Fecal pellets were homogenized with sterile ceramic beads by using the FastPrep-24 device (MP Biomedicals, Eschwege, Germany), followed by centrifugation and extraction of 140 µL of the supernatant.

Prior to further processing a maximum of 10 RNA extracts were pooled by taking 5 µL per sample to obtain a final volume of 50 µL. Pools were prepared per sample type, resulting in 18 OS pools, 13 US pools and 8 F pools. The pooled RNA samples were treated with DNase at 37 °C by using a TURBO DNA-free Kit (Invitrogen, Carlsbad, CA, USA), followed by RNA transcription into cDNA by using the SuperScript IV Reverse Transcriptase reagent (Invitrogen) and random hexamer primers. Second strand was synthesized by using the NEBNext® Ultra™ II Non-Directional RNA Second Strand Synthesis Module (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. dsDNA was purified by using the Agencourt AMPure XP bead system (Beckman Coulter Life Sciences, Krefeld, Germany) and the final DNA concentration was determined by using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Hennigsdorf, Germany). Sample libraries were sequenced on a HiSeq 2500 or a NextSeq 550 sequencing device (Illumina, San Diego, CA, USA) with a paired end read output of 2 × 250 bp (HiSeq) or 2 × 150 bp (NextSeq) and a total output of up to 8 million reads per pool. A detailed overview of the pools, included samples and read output is given in the supplementary material (Table ST1).

NGS data analysis and virome assembly

Prior to the NGS data analysis a quality trimming of sequencing reads was performed by using the tool Trimmomatic v0.39 [22]. For further analysis the diamond alignment tool was utilized to compare trimmed reads to the non-redundant protein virus database (NCBI, RefSeq release 210 from 3 January 2022) by using the BLASTx algorithm [12, 23]. The results were analyzed and the distribution of assigned viral reads per pool was evaluated in MEGAN [24]. This was also compared between the different sample types and among the pools of each sample type. Selected results were subsequently blasted to the whole NCBI database by using the BLASTn algorithm. For a number of hits the full genome sequences were downloaded and used as a reference for mapping the trimmed NGS data to identify more reads and to evaluate the

mapping quality visually in Geneious Prime software (version 2020.2.3, Biomatters Ltd., Auckland, New Zealand). Wherever possible, the nucleotide identities to related strains were calculated for the longest contig assembled on a conserved gene such as the RNA polymerase gene.

For viral sequences of high interest, suitable primers were designed on contigs supported by high read coverage. Subsequently, the presence of the novel viral sequences was confirmed in the initial sample pools by using conventional PCR under standard conditions (available on request). PCR products appearing as bands in the analytic agarose gel were purified and Sanger sequenced when sufficient quantity was reached.

Phylogenetic reconstruction

For selected viral sequences of interest, suitable contigs were used for phylogenetic reconstruction. For this purpose, a number of reference sequences were downloaded from NCBI database and a gap-free nucleotide alignment was calculated by using the MAFFT algorithm [25]. The phylogenetic trees were calculated based on this alignment by using the Bayesian MCMC algorithm (MrBayes version 3.2.6) [26]. A suitable model for each alignment was estimated by using the Akaike information criterion (AIC) prediction with the tool JModelTest [27, 28]. The calculation parameters were different depending on the virus and are specified in the results section (see figure legends Figure 4, Figure 5 and Figure 6), respectively.

Results

Following mNGS, the sequence reads were trimmed and analyzed separately per pool as described in the methods section. Comparison of the pools per sample type revealed mainly homogenous distributions of viral reads assigned to the respective viral orders (see supplemental Figures SF1–SF3). For further analysis, viral reads of all pools per sample type were combined and the assigned viral reads were compared between OS, F and US by using MEGAN as shown in Figure 2.

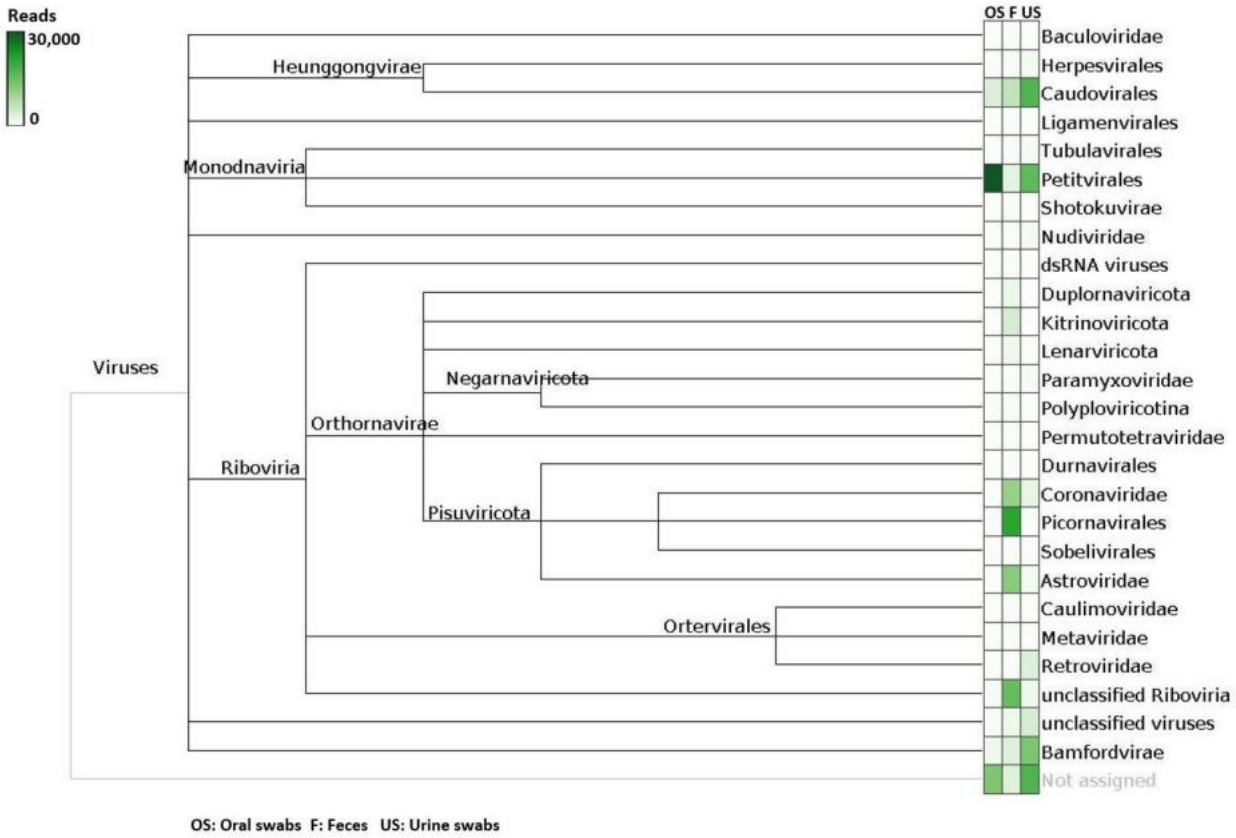

Figure 2. Normalized comparison of viral hits from different sample types in MEGAN after diamond BLASTx. Sample types are depicted in the following order: oral swabs (OS), feces (F), urine swabs (US). The intensity of green color represents the quantity of reads assigned to the respective viral family or order.

As shown in Figure 2, bacteriophages (e.g. *Caudovirales*, *Petitvirales*) were found in all sample types but in different amounts. For OS, no other viral hits of interest were assigned from the NGS data. Viral reads belonging to six different families (*Coronaviridae*, *Iflaviridae*, *Picornaviridae, Astroviridae, Paramyxoviridae* and unclassified *Riboviria*) were found in F samples and confirmed in silico and also via PCR. In the US samples, viral reads of three families (*Paramyxoviridae*, *Coronaviridae* and *Astroviridae*) were detected and confirmed in silico and also via PCR.

Some of the other preliminary results from BLASTx (depicted in Figure 2) could not be confirmed in the subsequent in silico analysis and were considered as possible cross-contaminations from the NGS run (e.g. *Herpesvirales* and *Bamfordvirae*) as commonly described [29]. An overview of the confirmed results after quality checks is given in Table 1, specifying details of the obtained NGS data, related virus and novel identified virus strains.

Table 1: Overview of the results obtained from mNGS data analysis of feces and urine swab samples from *M. fuliginosus* bats collected in July 2018. The table indicates the related virus, sample types and pool numbers from which the results were obtained, including the number of assigned reads, longest contig and nucleotide identity. The name and accession number of the novel virus strain as uploaded to GenBank are indicated, respectively.

	Astroviridae	
Name of related virus	**Bat astrovirus 1 isolate AFCD77 (EU847151)**	
Sample type	Feces samples	Urine swab samples
Pool numbers	F1, F2, F3, F4, F5, F6, F7, F8	U4, U8, U9, U14, U15
Assigned reads / longest contig	289 / 1068 nt	422 / 379 nt
Nucleotide identity	82 %	85.6 %
Name of novel virus strain	Bat astrovirus isolate F2/18 (Acc. OP141159)	Bat astrovirus isolate US2/18 (Acc. OP141166)
Name of related virus	**Mamastrovirus 14 isolate AFCD57 (NC_043099)**	
Sample type	Feces samples	
Pool numbers	F3	
Assigned reads / longest contig	1045 / 1366 nt	
Nucleotide identity	86.5 %	
Name of novel virus strain	Mamastrovirus 14 isolate F2/18 (Acc. OP141160)	
Name of related virus	**Mamastrovirus 18 isolate AFCD337 (NC_043102)**	
Sample type	Urine swab samples	
Pool numbers	U3, U5, U6, U9, U11, U12, U14	
Assigned reads / longest contig	282 / 311 nt	
Nucleotide identity	84.4 %	
Name of novel virus strain	Mamastrovirus 18 isolate US2/18 (Acc. OP141167)	
	Coronaviridae	
Name of related virus	**Bat alphacoronavirus isolate batCoV/MinFul/2018/SriLanka (OL956935)**	
Sample type	Feces samples	Urine swab samples
Pool numbers	F1, F2, F3, F4, F5, F6, F7, F8	U2, U3, U4, U5, U6, U8, U9, U12, U13, U14
Assigned reads / longest contig	994 / 729 nt	10,753 / 1226 nt
Nucleotide identity	83.1 %	98.6 %
Name of novel virus strain	Bat alphacoronavirus isolate MinFul/F2/2018/SriLanka (Acc. OP141161)	Bat alphacoronavirus isolate MinFul/U2/2018/SriLanka (Acc. OP141168)
Name of related virus	**BtMf-AlphaCoV/GD2012 (KJ473797)**	
Sample type	Feces samples	Urine swab samples
Pool numbers	F1, F2, F3, F4, F5, F7	U4, U6, U9, U12, U13, U14
Assigned reads / longest contig	2443 / 1113 nt	2182 / 769 nt
Nucleotide identity	83.1 %	85.5 %
Name of novel virus strain	Bat alphacoronavirus isolate AlphaCoV/F2/2018 (Acc. OP141162)	Bat alphacoronavirus isolate AlphaCoV/U2/2018 (Acc. OP141169)
	Iflaviridae	
Name of related virus	**Spodoptera exigua iflavirus 2 isolate Korean (JN870848)**	
Sample type	Feces samples	
Pool numbers	F1	
Assigned reads / longest contig	373 / 1283 nt	
Nucleotide identity	96.8 %	
Name of novel virus strain	Spodoptera exigua iflavirus isolate F2/18 (Acc. OP141163)	
	Paramyxoviridae	
Name of related virus	**Jingmen Miniopterus schreibersii paramyxovirus 1 (MZ328288)**	
Sample type	Urine swab samples	
Pool numbers	U2, U3, U6, U7, U9, U11, U12, U14	
Assigned reads / longest contig	573 / 450 nt	
Nucleotide identity	84 %	
Name of novel virus strain	Miniopterus schreibersii paramyxovirus isolate US2/18 (Acc. OP141170)	

	Picornaviridae
Name of related virus	**Miniopterus schreibersii picornavirus 1 (JQ814851)**
Sample type	Feces samples
Pool numbers	F1, F2, F3, F4, F5, F6, F7, F8
Assigned reads / longest contig	5714 / 3452 nt
Nucleotide identity	86 %
Name of novel virus strain	Miniopterus schreibersii picornavirus isolate F2/18 (Acc. OP141164)
	Unclassified Riboviria
Name of related virus	**Hubei sobemo-like virus 21 strain CC64469 (KX882813)**
Sample type	Feces samples
Pool numbers	F1, F3, F4
Assigned reads / longest contig	439 / 534 nt
Nucleotide identity	82.1 %
Name of novel virus strain	Hubei sobemo-like virus isolate F2/18 (Acc. OP141165)

For the families *Coronaviridae, Picornaviridae, Astroviridae* and *Paramyxoviridae*, the presence of viral sequences was furthermore confirmed in the original RNA pools (see Table 1) by using PCR and specifically designed primer based on the NGS data. The remaining viruses (*Iflaviridae* and unclassified *Riboviria*) were solely confirmed with in silico analysis of the data.

From all viral assemblies, either the longest contig or the contig used for phylogenetic reconstruction was uploaded to GenBank. These sequences of newly detected virus strains were named in relation to the closest related reference virus and with respect to current ICTV classification criteria (see Table 1).

Astroviridae

Astroviruses (n=3) were found in F and US samples. In F samples, 1045 reads were assigned to Mamastrovirus 14 isolate AFCD57 (NC_043099) with a nucleotide identity of 86.8 % on the longest contig (1366 nt), located overlapping on the polyprotein 1AB and capsid protein CDS. Further 289 reads from F samples were assembled to Bat astrovirus 1 isolate AFCD77 polyprotein 1AB gene (EU847151) with a nucleotide identity of 82 % on the longest contig (1068 nt).

A total of 422 reads from US samples were also assembled to EU847151, with a nucleotide identity of 85.6 % on the longest contig (379 nt).

From US samples further 282 reads were assembled to Mamastrovirus 18 isolate AFCD337 (NC_043102) with a nucleotide identity of 84.4 % on the longest contig (311 nt), located on the polyprotein 1AB gene.

Because of the lack of overlapping sequences, phylogenetic reconstruction was calculated exemplarily with Bat astrovirus isolate F2/18 (OP141159); the results are shown in Figure 3.

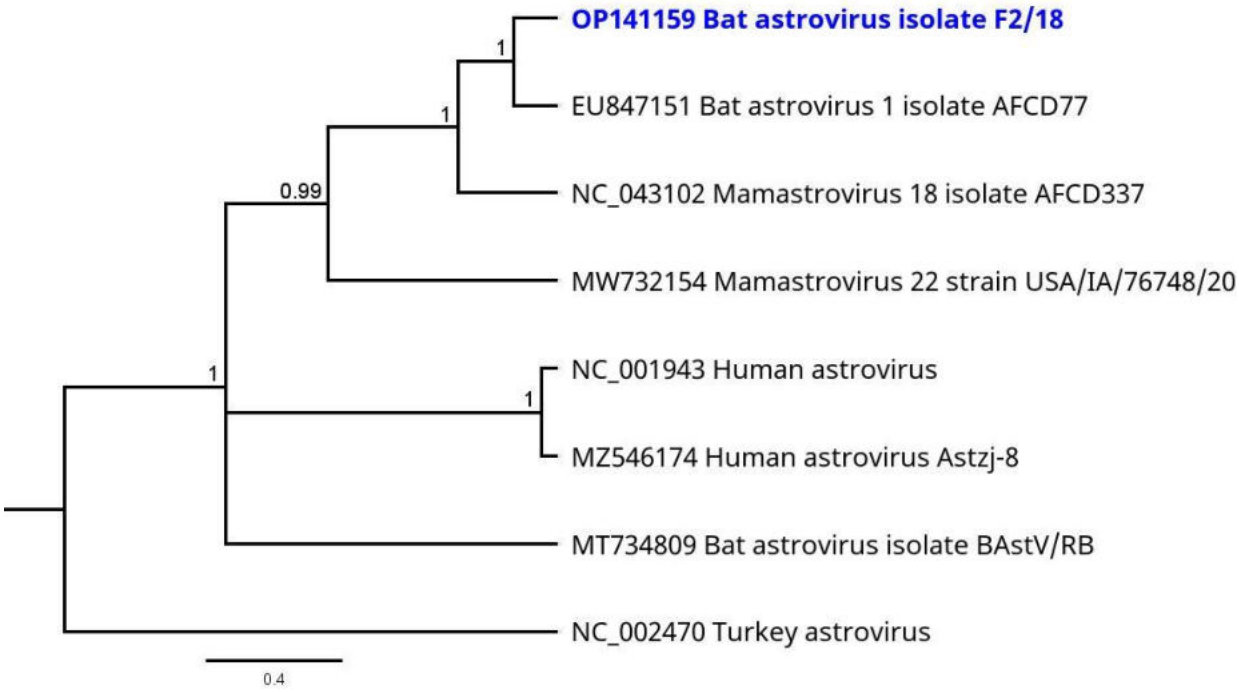

Figure 3: Phylogenetic tree based on the mNGS sequences obtained from the virome of F sample pools (highlighted in blue) and a selection of other astrovirus species. Turkey astrovirus (NC_002470) was used as outgroup. Phylogenetic reconstruction was calculated with the Bayesian MCMC algorithm: 500,000 generations were calculated with a subsampling frequency of 100 and a burn-in of 10 %. The substitution model GTR was selected with a gamma-distributed rate variation. Visualized as molecular clock with uniform branch lengths.

Coronaviridae

Two coronaviruses (CoV) were found in US and F samples, respectively. A total of 994 reads from F samples and 10,753 reads from US samples were mapped to the bat alphacoronavirus isolate batCoV/MinFul/2018/SriLanka (OL956935) with nt identities on conserved ORF1B CDS of 80.36 % (F, 668 nt contig) and 98.37 % (US, 735 nt contig). In addition, 2443 reads from F sample pools and 2182 reads from US sample pools were mapped to BtMf-AlphaCoV/GD2012 (KJ473797), an alphacoronavirus HKU8 strain from China. Nucleotide identities on the conserved ORF1B CDS were calculated with 82.9 % (F, 1113 nt contig) and 85.5 % (US, 769 nt contig). For phylogenetic reconstruction, contigs on the ORF1B CDS were selected, respectively. Because of the lack of overlapping contigs, phylogeny was calculated separately for the sequences from F and US samples but by using the same reference strains including common human pathogenic CoVs. For the conserved ORF1B CDS, overlapping contigs of 182 nt (F samples) and 224 nt (US samples) were obtained. Figure 4 shows the phylogenetic reconstruction of CoV from US sample pools.

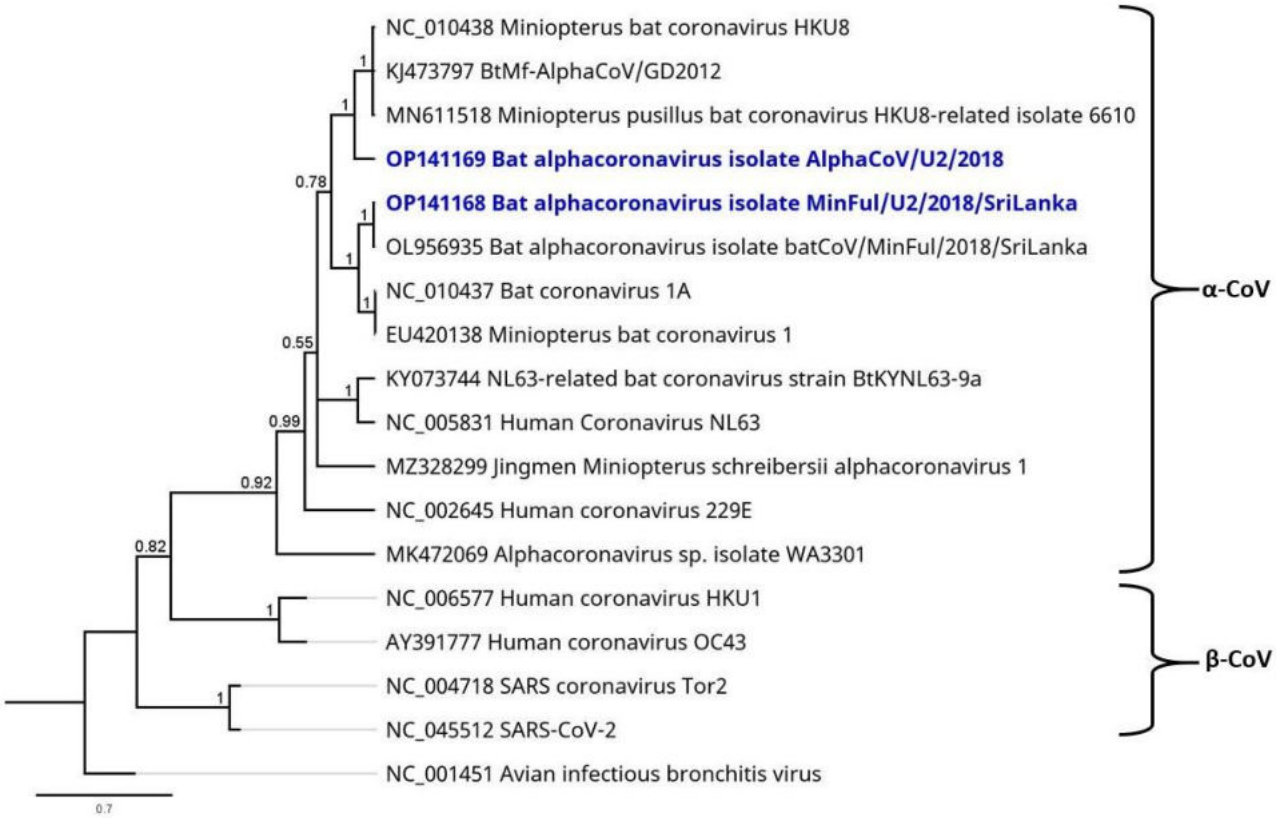

Figure 4: Phylogenetic tree based on the mNGS sequences obtained from the virome of US sample pools (highlighted in blue) and a selection of α-CoVs and β-CoVs as specified. For use as outgroup, the γ CoV avian infectious bronchitis virus (NC_001451) was also included in the calculation. Phylogenetic reconstruction was calculated with the Bayesian MCMC algorithm: 1,000,000 generations were calculated with a subsampling frequency of 100 and a burn-in of 10 %. The substitution model GTR was selected with a gamma-distributed rate variation. Visualized as molecular clock with uniform branch lengths.

The calculation confirms the presence of two different strains that are allocated to different branches inside the genus *Minunacovirus*. The identified Bat alphacoronavirus batCoV/MinFul/2018/SriLanka US2/2018 clusters with strains of the species Miniopterus bat coronavirus 1, whereas the other Miniopterus AlphaCoV strain US2/2018 clusters with HKU8-related strains.

Iflaviridae

An iflavirus was found in F samples with a total of 373 reads assembled to Spodoptera exigua iflavirus 2 isolate Korean (JN870848), covering 93.6 % of the genome. The longest contig of 1283 nt, located at the beginning of the polyprotein CDS, shares a nucleotide identity of 96.8 % to the related Spodoptera exigua iflavirus 2.

Paramyxoviridae

A paramyxovirus (PMV) was found in US samples and confirmed by PCR (compare Table 1). A total of 573 reads were mapped to the full genome of Jingmen Miniopterus schreibersii paramyxovirus 1 (MZ328288). The longest contig (450 nt) on the conserved L gene showed highest nucleotide identities to this strain (84 %) and to the partial genome of Miniopterus schreibersii paramyxovirus isolate Bat Ms-ParaV/Anhui2011 (KC154054; 84.6 % nt identity). This contig was also selected for phylogenetic reconstruction. The phylogenetic tree of 16 paramyxoviruses including the novel sequence from Sri Lanka and selected human pathogenic strains is shown in Figure 5.

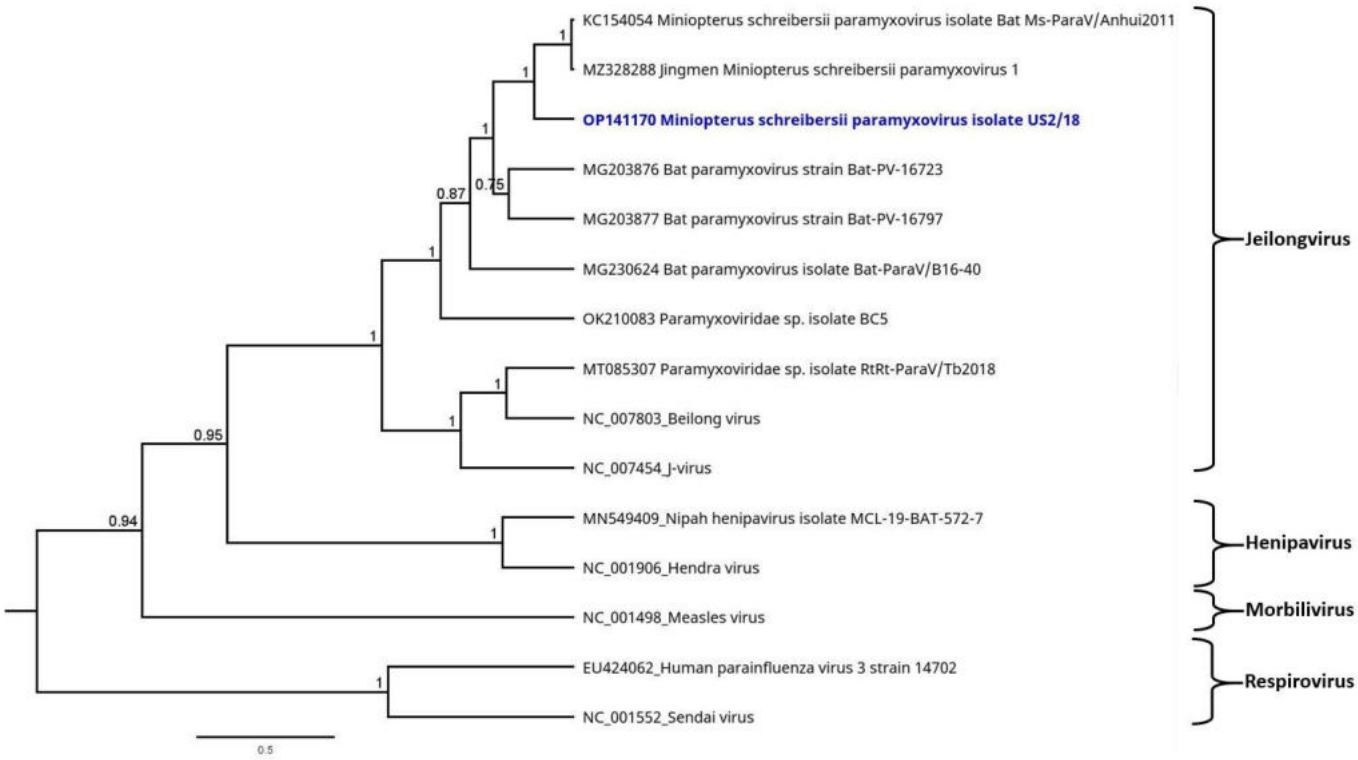

Figure 5: Phylogenetic tree based on the mNGS sequences obtained from the virome of US sample pools (highlighted in blue) and a selection of PMVs. Sendai virus (NC_001552) was selected as outgroup for the calculation. Phylogenetic reconstruction was calculated with the Bayesian MCMC algorithm: 1,000,000 generations were calculated with a subsampling frequency of 100 and a burn-in of 10 %. The substitution model GTR was selected with a gamma-distributed rate variation. Visualized as molecular clock with uniform branch lengths.

The phylogenetic reconstruction confirms that the novel paramyxovirus from Sri Lanka is closely related to the two PMV strains as described before. Other Miniopterus-related PMVs from China cluster in the same branch of the tree, representing the subgenus Jeilong virus. Apart from this, the subgenera Henipavirus, Morbillivirus and Respirovirus were allocated to distinct branches of the tree, respectively. These subgenera also include the human pathogenic strains that were selected for this phylogenetic reconstruction.

Picornaviridae

A Picornavirus was found in F samples, a total of 5714 NGS reads were assembled to Miniopterus schreibersii picornavirus 1 (JQ814851), covering most parts of the genome. The longest contig of 3452 nt, located at the end of the polyprotein CDS, shares a nucleotide identity of 86 % to the Miniopterus schreibersii picornavirus 1. For phylogenetic reconstruction, a well-covered contig of 311 nt was selected on the highly conserved 2C peptide on the picornaviral polyprotein. The phylogenetic reconstruction is illustrated in Figure 6.

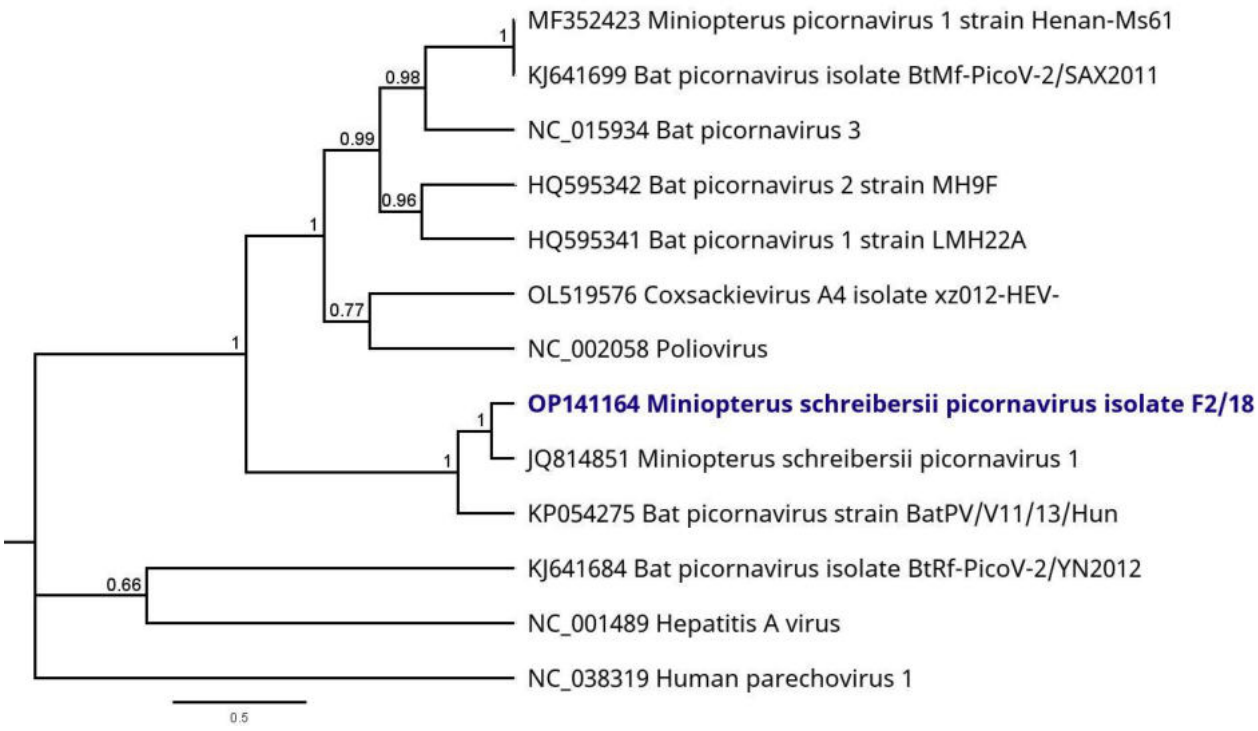

Figure 6: Phylogenetic tree based on the mNGS sequences obtained from the virome of F sample pools (highlighted in blue) and a selection of picornaviruses. Human parechovirus 1 (NC_038319) was selected as outgroup for the calculation. Phylogenetic reconstruction was calculated with the Bayesian MCMC algorithm: 1,000,000 generations were calculated with a subsampling frequency of 100 and a burn-in of 10 %. The substitution model GTR was selected with a gamma-distributed rate variation. Visualized as molecular clock with uniform branch lengths.

As shown in Figure 6, the novel bat picornavirus strain from F samples clusters monophyletically with its closest related strain Miniopterus schreibersii picornavirus 1 and the bat picornavirus strain BatPV/V11/13/Hun, both obtained from *Miniopterus schreibersii* bats. Other bat- and Miniopterus-hosted picornaviruses were allocated to different branches of the phylogenetic tree. The human pathogenic representative picornaviruses are clearly distant from the novel Sri Lankan strain.

Unclassified Riboviria

From F samples, 439 reads were assembled to a Hubei sobemo-like virus 21 strain CC64469 (KX882813) with a nucleotide identity of 82.1 % on the longest contig (534 nt).

Phages

A number of phages were found in each sample type as described. Considerable amounts of reads were assigned to the bacteriophage orders *Caudovirales* (OS: 576; F: 1278; US: 663,683) and *Petitvirales* (OS: 44,741; F: 235; US: 554,119), respectively.

Discussion

In our study we analyzed the virome of *M. fuliginosus* bats inhabiting the Wavul Galge cave in the interior of the island of Sri Lanka. By taking orals swabs, urine swabs and feces, we aimed to examine if different viruses are shed by distinct shedding routes represented by the individual sample types. This assumption was confirmed and we have been able to obtain different virome compositions for each sample type.

In oral swab samples, the primary viral hits were assigned to phages belonging to the orders *Caudovirales* and *Petitvirales*. The presence of any other viral sequences in oral swab samples was not confirmed after quality assessment of analyzed NGS data. However, the detection of *Caudovirales* and *Petitvirales* in OS serves as proof of principle also for this sample type.

Apart from this, different other viruses were detected in the feces and urine swab samples as discussed in the following sections.

Phages and viruses from other presumed reservoir hosts

Sequence reads assigned to phages were found in high numbers in all three sample types. Although these were not inspected in detail in this study, the large number and variety of reads also indicates the presence of numerous bacteria in the collected samples. In combination with 16S bacterial metagenomic analysis, these results will be interesting to be further explored in future studies.

In feces samples, viral sequences were identified matching Hubei sobemo-like virus 21 strain CC64469 (China), belonging to unclassified *Riboviria*. This virus was originally isolated from invertebrates within the phylum *Annelida* [30]. Most probably a worm carrying this virus was taken in as nutrition by the bat, passaged and excreted with the feces afterwards.

In addition to this, viral sequences assigned to Spodoptera exigua iflavirus 2 (Korea) were identified in feces samples. This virus belonging to the family *Iflaviridae* within the order *Picornavirales* was isolated from insects of the genus *Spodoptera* (moths) [31]. Here again it is very likely that the virus was taken in with moths for nutrition, passaged and excreted afterwards. In both examples, it is impossible to conclude whether the virus infected the bats as well or was merely digested, passaged and excreted. For this, tissues and organs would need to be investigated.

However, the identified phages as well as these two examples show that the viromes derived from NGS data are complex and do not only comprise the bat-related viruses. Moreover, the data also reveal a number of other viruses derived from the inherent bacterial flora within the bat as well as viruses derived from insects serving as nutrition for the bats. This in turn has the potential to analyze virome compositions of whole habitats, including different organisms. This may be the basis or part of further investigations regarding the bacterial flora and dietary habits of the bats.

Astroviridae

The viruses within the family *Astroviridae* are common in a wide range of birds (genus *Avastroviruses*) and mammals (genus *Mamastrovirus*), including bats and humans [32]. Human astrovirus infections mainly cause gastroenteritis in children [33]. Members of this family have a high genetic diversity depending on the respective host species and a wide host range (birds, mammals). However, the zoonotic potential of bat-hosted astroviruses is widely unexplored as yet [34]. Members of the mamastrovirus genus are classified based on the amino acid sequences of the capsid region and can be further divided into species depending on the host and other genetic criteria [35]. Based on the analyzed sequence data, the examined bats carried multiple astrovirus strains. With this, bat astrovirus sequences were detected for the first time in a bat species from Sri Lanka, namely *M. fuliginosus*.

From US samples, Bat astrovirus isolate US2/18 (OP141166) and Mamastrovirus 18 isolate US2/18 (OP141167) were identified, whereas from F samples Bat astrovirus isolate F2/18 (OP141159) and Mamastrovirus 14 isolate F2/18 (OP141160) were identified. All three viruses were originally detected in *Miniopterus* bat species, which may indicate a high host specificity of these viruses. In addition, the circulation of different bat astrovirus strains within one bat population is conceivable, as already reported in other studies [36–38].

Phylogenetic reconstruction allowed for a rough classification of the Bat astrovirus isolate F2/18 from Sri Lanka, as an example. Although only few suitable reference strains were available in the databases, the phylogeny distinguished astroviruses of bats from astroviruses of other vertebrates (turkey, human) by allocation to different tree branches.

Most available bat astroviral references from the databases contained partial genomes only, located at different regions. Further sequence analysis and phylogenetic comparison between all newly obtained astrovirus sequences from Sri Lanka were therefore not possible with the available data. Consequently, we cannot finally prove the presence of multiple astrovirus strains in the collected samples. It may be possible that the sequences are originally derived from a single astrovirus but were mapped to the different partial genomes obtained from the database. Therefore, virus isolation of these viruses followed by in-depth sequencing of the missing genome sequences could be helpful in order to obtain full genome data or at least full gene sequences. With further sequence information it could be examined whether the astroviral reads were actually derived from different strains or if they belonged to a single astrovirus within the samples.

Coronaviridae

Presence of two different CoVs in *M. fuliginosus* bats was confirmed in F and US samples. Both related virus strains were originally detected in *Miniopterus* bats [20, 39, 40]. The CoV full genome from Sri Lanka (OL956935) was derived from rectal swabs collected during the same bat sampling session as this study and reported previously [20]. With the virome sequence data of F and US samples in this study, we were able to confirm the presence of this virus strain in other sample types. In addition, we identified Bat alphacoronavirus isolate AlphaCoV/F2/2018 and Bat alphacoronavirus isolate AlphaCoV/U2/2018 which are closely related to Miniopterus bat coronavirus HKU8 strains. All identified viruses belong to *Minunacoviruses*, an α-CoV subgenus containing *Miniopterus*-hosted bats. The slight differences between the two virus species within the subgenus are also visible in the phylogenetic reconstruction of different CoVs. Representative strains of the virus species Miniopterus bat coronavirus 1 and Miniopterus bat coronavirus HKU8 were divided into two different clusters within the branch of *Minunacoviruses*. Co-existence of these two virus species and different CoVs in general have already been reported before [41–43]. Therefore, it can be assumed that these two or even more CoV strains circulate steadily in this population of *M. fuliginosus* bats. Regarding the zoonotic potential of the detected CoVs, further investigation of full genes and specific receptor-binding domains would be necessary for more precise

statements. Based on the available data and phylogenies calculated from this, we did not find indications for a human pathogenic potential of the detected viruses [17, 20]. Human pathogenic CoVs that cause outbreaks and pandemics like SARS, MERS and Covid-19 all belong to the genus of β-CoV and are genetically diverse from the genus α-CoV. Although mildly human pathogenic viruses such as HCoVs NL63 and 229E are represented in the α-CoV genus as well, the phylogeny ranks these species as rather distantly related to the *Minunacoviruses*.

Paramyxoviridae

Representatives of *Chiroptera*-hosted PMVs are able to cause zoonotic diseases in humans; therefore this virus family was of high interest for the virome analysis of the bat samples [44]. The detection of PMVs in US samples was expected, as this is the usual shedding route of these viruses [45]. The presence of PMVs in the collected US samples was also detected via semi-nested PCR [7] and Sanger sequencing as reported before [18]. These results indicated the presence of multiple PMVs in the bat population. The co-circulation of multiple PMVs is common in bats and has been reported before [46, 47]. With the obtained NGS data of this study we were able to confirm PMVs in the samples by using another molecular virus detection method. With this, a proof of principle of the methodical approach was possible. However, the NGS data did not reveal enough sequence information to confirm multiple PMV strains. For this purpose, NGS of single US samples instead of pools and comparison of sequence data between individual bats will be necessary.

As expected, the identified Miniopterus schreibersii paramyxovirus isolate US2/18 (OP141170) has the highest similarities to other *Miniopterus*-hosted PMVs. In the phylogenetic tree, all *Miniopterus*-derived strains are assigned to the group of Jeilong virus (Figure 5). The other branches of the phylogenetic tree depict only a small number of representative strains, including human pathogenic PMVs, whereas the actual *Orthoparamyxovirinae* subfamily is notably more diverse [44, 48]. In accordance to this, different PMVs can cause diseases of different severity in humans (e.g. Human parainfluenzavirus vs. Nipah virus), whereas other PMVs have a rather low zoonotic potential. The preliminary analysis of the limited sequence data and the phylogenetic reconstruction give no indication of a human pathogenic potential of the novel PMVs. Further sequence data will be needed to allow for a detailed and complex analysis and taxonomic classification of the novel PMV strain [49].

Picornaviridae

The family of picornaviruses is a highly diverse family with 68 genera and 158 virus species according to ICTV [35]. They are globally distributed in a number of bat species including *Miniopterus* bats [50, 51]. Additionally, they are found in a number of other host species including birds, livestock and humans. Cross-species transmissions between different bats or mammals are possible as well as zoonotic transmissions to humans [52]. In humans, picornaviruses such as enterovirus, rhinovirus, coxsackievirus, hepatovirus A and human parechovirus can cause diseases of the nervous system and the respiratory and gastrointestinal tracts [53].

A number of sequences related to Miniopterus schreibersii picornavirus 1 were identified in feces samples, which is a described shedding route of bat picornaviruses [54]. The novel strain from Sri Lanka Miniopterus schreibersii picornavirus isolate F2/18 (OP141164) shares an identity of 86 % to the reference strain from China [55]. For phylogenetic reconstruction in this study, a suitable sequence on the 2C peptide was selected which is a highly conserved region on the picornaviral polyprotein and therefore suitable for this analysis [56]. The phylogenetic reconstruction included several bat picornaviruses and human pathogenic strains. The novel picornavirus strain from Sri Lankan *M. fuliginosus* bats was assigned to a branch with other *Miniopterus*-hosted picornaviruses, and the human pathogenic species were assigned to other branches of the tree. The available results did not indicate a human pathogenic potential of the identified picornavirus. Although the phylogenetic analysis was limited to a small proportion of the *Picornaviridae* family, we were able to get a general idea on phylogenetic relationships of the novel bat picornavirus from Sri Lanka. For proper species classification, a full protein sequence analysis of P1, 2C, 3C and 3D proteins will be necessary but was not possible from the obtained data. However, the results represent the first detection of a picornavirus in the bat species *M. fuliginosus* from Sri Lanka.

Conclusion

We were able to analyze the different compositions of the *M. fuliginosus* virome by potential shedding route obtained from oral swabs, urine swabs and feces samples. Depending on the sample types, different viruses were detected via NGS analysis, corresponding to their typical shedding routes, respectively.

Independent of the sample type, we were able to detect the co-existence of astroviruses, coronaviruses, paramyxoviruses and picornaviruses circulating simultaneously in the *M. fuliginosus* bat population. Co-existence of these viruses may be common in bats, and even a co-speciation of virus species with their specific host is discussed [57, 58]. It is assumed that also virus–virus interaction is possible and may influence the host, resulting in very specific viral shedding patterns depending on the virome composition [47]. In future, the epidemiological consequences of co-existing viruses in the bats should be further examined.

It is remarkable that mainly bacteriophages were identified in oral swab samples, although saliva is also known as common shedding route for other virus families. Lyssaviruses including rabies-related viruses would have been most likely to be found in this sample type. If these viruses are prevalent in the bats they are excreted by salivary glands and therefore shed with the saliva [59]. However, the excretion of viruses is generally affected by seasonal patterns and may have been low at the respective sampling point. Since we only used non-invasive sampling methods and could not examine bat brain or other tissue samples, we cannot conclude whether or not such viruses were prevalent in bat organs at the time of bat sampling. A long-term and frequent bat sampling will help to understand seasonality and shedding patterns of different viruses of interest. Probably the virome of all different sample types would change over time as it is influenced by seasons and environmental factors like temperature, humidity, rainfall, migration and reproduction cycles [1, 47, 60, 61].

In general, all collected sample types also represent possible transmission routes from bats to humans. The way of viral shedding depends on the respective tissue where the virus replicates: e.g. replication in kidneys and shedding via urine or replication in intestine organs and shedding via feces [62].

Transmission of viruses via saliva would be possible from bites when catching and handling the bats. Urine is constantly shed by the bats in the Wavul Galge cave, and the intake of these aerosols containing viral particles may lead to virus transmission when entering the cave without any protective equipment [63]. In this context, it would also be of interest in the future to investigate the virome of the other bat species inhabiting the cave and to conclude whether they are susceptible for the same viruses as well.

Feces is also constantly shed by the bats in their roosting cave. Although the fecal–oral transmission route is rather unlikely, local people are in close contact to bats when collecting bat guano to use as organic fertilizer [64]. Especially in rural areas like those around the Wavul Galge cave, the use of bat guano in agriculture is common and represents a potential

transmission risk to the farmers. Although our study results revealed virus species of rather low zoonotic potential, this does not exclude the seasonal presence of potentially pathogenic agents. A special awareness regarding possible transmissions should be raised. Concurrently, the fear of zoonotic viruses in bat hosts should not justify their eradication. On the contrary, the natural habitat of the bat population in the cave should be recognized and respected.

In summary, the virome composition of different sample types obtained from *M. fuliginosus* bats in Sri Lanka was analyzed for the first time. Recent DNA barcoding and morphological studies on this species suggest that the *Miniopterus* bats inhabiting the island of Sri Lanka are in fact a new species of bat, not described hitherto and named as *Miniopterus phillipsi* [65]. Based on these findings, the results from our work would represent the first virome analysis for this newly described bat species.

Acknowledgement

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Comparative virome analysis of individual shedding routes of Miniopterus fuliginosus *bats inhabiting the Wavul Galge Cave, Sri Lanka*

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Supplementary Table

Table ST1: Overview of the sample pools for NGS analysis, specifying included samples, total number of obtained reads after trimming and total reads that were assigned to viruses in MEGAN.

Sample type	Pool	Included samples (Bat no.)	Total reads after trimming	Total reads assigned to viruses (MEGAN)
Oral swabs	O1	85, 87, 88, 89, 91, 94, 95, 96, 98, 100	4,906,956	3,005
	O2	101, 103, 104, 106, 107, 108, 109, 110, 111, 113	5,381,629	1,757
	O3	114, 115, 116, 117, 118, 119, 120, 121, 122, 123	5,416,413	3,317
	O4	124, 125, 126, 127, 128, 129, 130, 131, 132, 133	4,568,104	1,732
	O5	134, 135, 136, 137, 138, 139, 140, 142, 143, 144	4,609,884	1,776
	O6	145, 146, 147, 148, 149, 150, 152, 153, 154, 156	6,206,508	2,584
	O7	157, 158, 159, 160, 161, 162, 163, 164, 165, 166	4,984,406	2,731
	O8	167, 168, 169, 170, 171, 172, 173, 174, 175, 176	4,570,809	1,153
	O9	177, 178, 179, 180, 181, 182, 183, 184, 185, 186	3,241,858	2,412
	O10	187, 188, 189, 190, 191, 192, 193, 194, 195, 196	4,515,414	1,629
	O11	197, 198, 199, 200, 201, 202, 203, 204, 205, 206	3,963,803	3,142
	O12	207, 208, 209, 210, 211, 212, 213, 214, 215, 216	3,794,828	1,578
	O13	217, 218, 219, 220, 221, 222, 223, 224, 225, 226	4,025,694	1,881
	O14	227, 228, 229, 230, 231, 232, 233, 234, 235, 236	4,549,502	2,165
	O15	237, 238, 239, 240, 241, 242, 243, 244, 245, 246	3,726,854	2,247
	O16	247, 248, 249, 250, 251, 252, 253, 254, 255, 256	5,625,662	1,465
	O17	257, 258, 259, 260, 261, 262, 263, 264, 265, 266	8,428,422	4,434
	O18	267, 268, 270, 271, 272, 273, 274, 275, 276	6,145,989	1,769
	O19	277, 278, 279, 280, 281, 282, 283, 284	3,905,991	2,243
Urine swabs	U2	87, 95, 96, 100, 101, 103, 104, 106, 111, 113	5,772,514	19,022
	U3	114, 116, 227, 118, 119, 120, 132, 134, 135	4,231,010	99,135
	U4	136, 137, 139, 143, 146, 147, 148, 155, 159, 160	3,626,749	80,589
	U5	156, 166, 167, 168, 169, 170, 171, 172, 175, 176	5,557,808	113,844
	U6	177, 178, 189, 181, 183, 184, 186	6,366,695	133,523
	U7	187, 189, 190, 191, 193, 195, 196	9,849,006	209,126
	U8	197, 198, 199, 201, 202, 203, 204, 205, 206	9,274,541	200,733
	U9	207, 209, 217, 218, 225	8,084,957	252,039
	U10	227, 229, 230, 232, 234, 236	4,264,597	90,724
	U11	239, 240, 241, 242, 244, 246	3,652,926	76,205
	U12	247, 248, 249, 253, 254, 255, 256	4,937,283	11,959
	U13	258, 260, 261, 262, 263, 264, 265, 266	3,537,353	71,699
	U14	267, 268, 270, 271, 272, 273, 278, 281, 284	922,687	17,963
Feces	F1	91, 94, 100, 101, 113, 119, 123, 125, 127, 128	2,847,745	4,079
	F2	131, 134, 142, 143, 147, 148, 149, 150, 153, 155	2,937,757	2,532
	F3	156, 161, 173, 179, 180, 182, 183, 186, 189, 192	3,454,655	15,268
	F4	195, 200, 202, 203, 204, 208, 209, 213, 215	2,404,303	3,173
	F5	218, 220, 221, 222, 226, 227, 228, 230, 233, 234	2,921,352	2,426
	F6	235, 236, 237, 238, 239, 241, 242, 243, 244, 245	1,497,502	882
	F7	246, 247, 249, 250, 251, 252, 254, 256, 257, 258, 259	2,564,216	749

Supplementary Table

	F8	261, 263, 268, 270, 279, 280, 282, 283	2,993,081	898

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Supplementary Figures

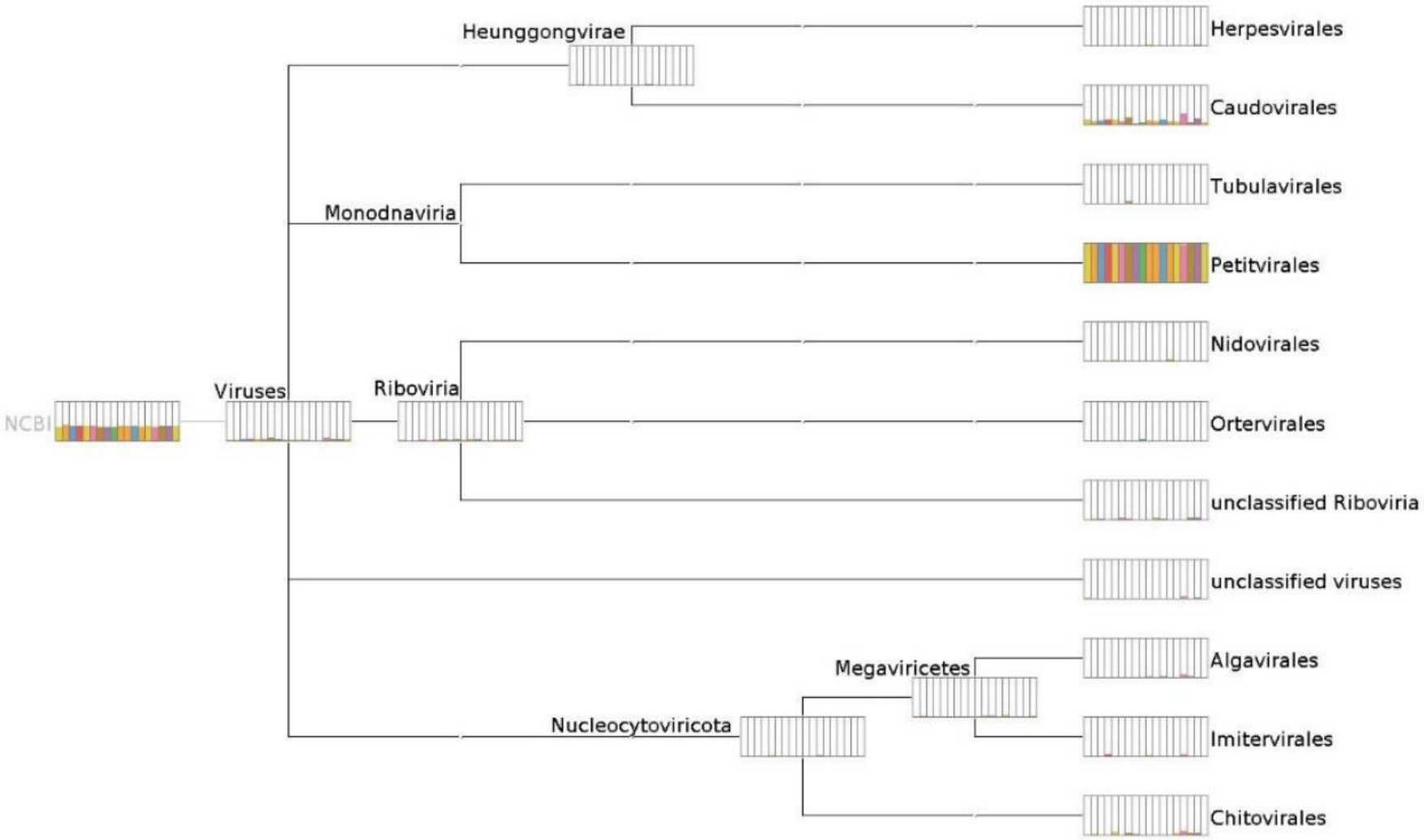


Figure SF1: Normalized comparison of viral hits obtained after mNGS from OS pools OS2.1 – OS2.19, analyzed with diamond BLASTx algorithm and visualized in MEGAN software.

Supplementary Figures

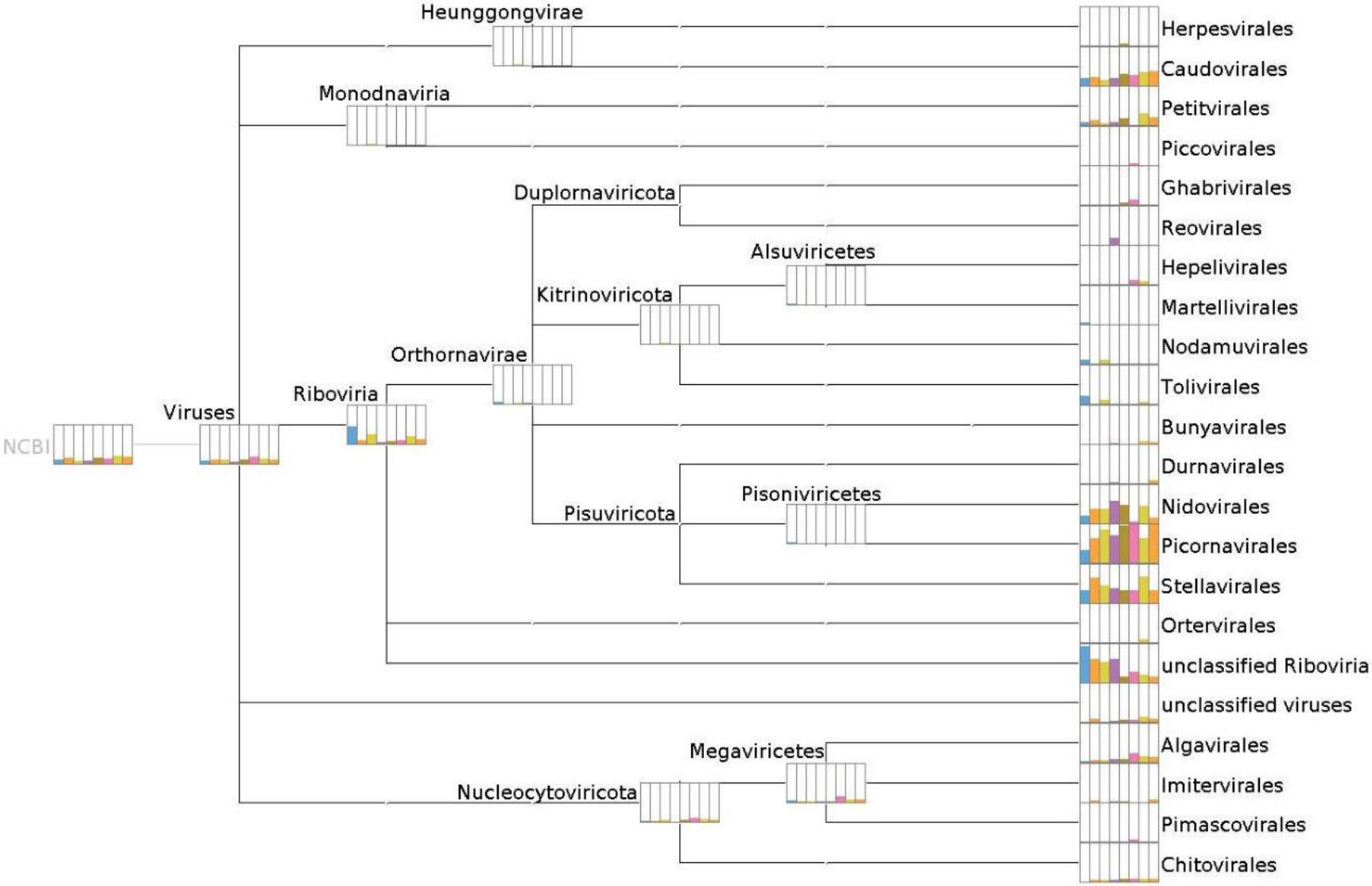
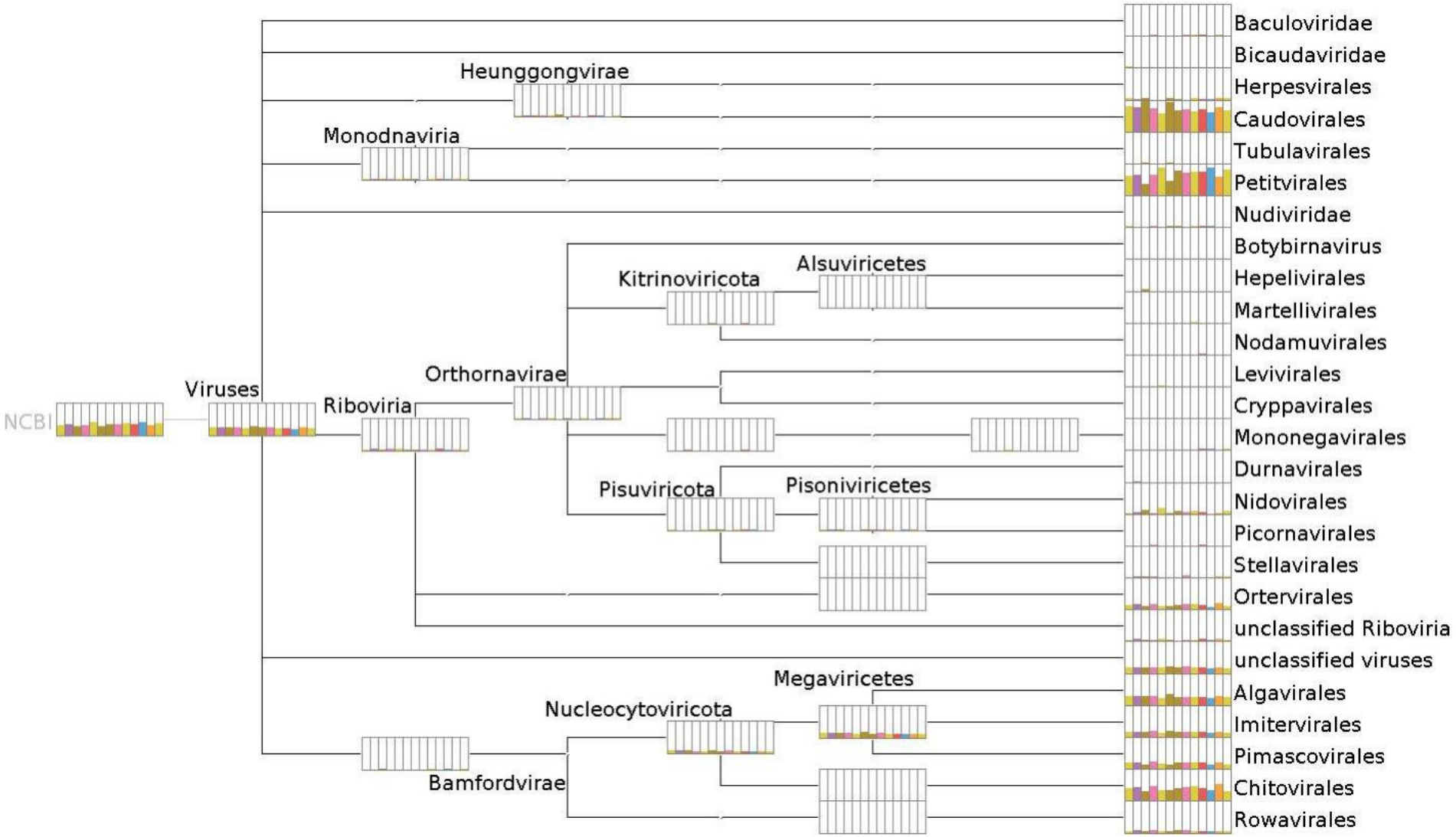


Figure SF2: Normalized comparison of viral hits obtained after mNGS from F pools F2.1 – F2.8, analyzed with diamond BLASTx algorithm and visualized in MEGAN software.

Supplementary Figures



47 *Figure SF3: Normalized comparison of viral hits obtained after mNGS from US pools US2.1 – US2.14, analyzed with diamond BLASTx algorithm*
48 *and visualized in MEGAN software.*