Virus diversity and cross-species transmission of viruses from the straw-coloured fruit bat *Eidolon helvum*

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"For to be free is not merely to cast off one's chains, but to live in a way that respects and enhances the freedom of others."
Nelson Mandela

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1. Introduction

1.1. Zoonosis and emerging diseases

The World Health Organization (WHO) defines zoonosis as "any disease or infection that is naturally transmissible from vertebrate animals to humans and vice-versa". Zoonotic agents may be viruses (Rabies virus), bacteria (*Salmonella* spp.), protozoa (*Toxoplasma gondii*) and helminths (*Fasciola* spp.). A disease is defined as emerging when it is "newly recognized or evolved, or has occurred previously but shows an increase in incidence or expansion in geographical, host or vector range". The increasing discovery of zoonoses is often related to better diagnostic tools, but the main causes of their emergence are human behaviour and modifications of natural habitats. Animals, particularly wild animals, are thought to be the source of >70% of all emerging infections [1] of which 25% are of viral origin [2]. Expansion of human population results in encroachment into undisturbed habitats which may lead to increased exposure to wildlife and their associated pathogens. The disturbance of habitats by humans inevitably leads to a loss of biodiversity, which may indirectly increase the possibility of emerging diseases [3]. This phenomenon has been described as the "dilution effect", postulating that a decrease in a host diversity leads to an increase of prevalence of infectious diseases and vice versa [4]. Furthermore, factors such as increased wildlife trade, live animal and bushmeat markets, and consumption of bushmeat provide an interface for pathogen transmission [5]. Additionally, globalization and associated increased global travel facilitate the global distribution of emerging pathogens within a few days [6]. Zoonotic viruses can be highly pathogenic for humans, however, the underlying factors that enable viruses to cross the species barrier are not known. In general, three factors are necessary for the establishment of a zoonotic virus. The host must be susceptible to the virus, the environmental conditions must provide stability and viability of the virus and the host, and the virus must come into contact frequently enough for a successful transmission [7]. It is believed that genetic relatedness of species favours cross-species transmission of pathogens [6, 8] but the intrinsic principles of these phenomenon are still not understood. For a successful transmission, viruses have to overcome ecological and molecular species barriers as, for example the virus entry by species-specific receptors. Even after the crossing of

receptor-dependent barriers, genome replication, gene expression and morphogenesis have to adapt to new intracellular environments. Moreover, the innate immunity of the new host needs to be evaded to establish a successful replication [9, 10]. Viruses with a broad host range can use different host cell mechanisms for replication and are therefore more likely to gain access to new hosts than viruses which are specialized in a single or closely related host [6]. Furthermore, it has been shown that it is more likely for a virus to adapt to humans when it has a broad range of life cycles and replication modes [11]. Another important factor are the transmission patterns of viruses which play an important role in the definition of ecological species barriers. Direct zoonotic virus transmission, for instance, can occur by saliva from reservoir animals, as in the case of rabies. More often viruses use vectors or intermediate amplifying hosts. Arthropod-borne viruses, like Alpha-, Bunya-, or Flaviviruses, are transmitted to humans via insects or ticks, which take up the virus when feeding on infected animals. Intermediate or amplifying hosts serve as bridges between two species, possibly facilitating stepwise adaptation and/or bringing the virus into contact with recipient hosts [6]. For example, Nipah virus is maintained in a bat reservoir, but use pigs as an amplifying host prior to transmission to humans [12]. The majority of the recently emerged zoonotic diseases were caused by RNA viruses. In comparison to DNA viruses, RNA viruses have an error-prone replication, insufficient or complete lack of proof-reading mechanisms and a short generation time [13]. These characteristics result in a more rapid genetic evolution of RNA viruses, which is believed to be crucial for successful transmission to a new host. Thus, cross-species transmission is more likely to happen if the virus has a RNA genome than a DNA genome.

Bats are increasingly recognized as sources of emerging zoonoses and harbour a variety of highly virulent RNA viruses including Rabies virus, Ebola- and Marburg virus, severe acute respiratory syndrome (SARS) virus, Hendra- and Nipah virus. The question of whether bats are special in their potential to harbour zoonotic viruses is widely discussed [14-16]. A number of characteristics may enhance their suitability as virus reservoirs. Bats account for 20% of all mammals and live on all continents except Antarctica. They can live in large social groups with a high population density, have a relatively long lifespan, they often live in sympatry, leading to a greater interspecific

transmission and are mobile [15-17]. Viruses in bat populations exhibit significantly genetic diversity and there is a theory that bats have ancient relationships with these viruses and hence serve as reservoir.

1.2. *Eidolon helvum*

Eidolon helvum (*E. helvum*), the straw-coloured-fruit bat, is the second largest fruit bat on the African continent and belongs to the family *Pteropidae* [18]. *E. helvum* is highly abundant in Sub-Saharan Africa with their primary habitat in the tropical forest and savannah. Their habitat stretches from Senegal in the west, across central Africa to Ethiopia in the east and down to South Africa in the south (Fig. 1). Colonies have also been recorded on several off-shore islands in the Gulf of Guinea, Zanzibar, Pemba and Mafia, on the Arabian Peninsular and has been sighted in Yemen and Saudi Arabia [18-20]. *E. helvum* form large colonies with up to 1 Million animals which use the same roosts and foraging areas over many years [21]. Each year, animals disperse into smaller colonies and migrate up to 2000 km along a south-north, north-south route following the rainfall gradient [18, 19, 22, 23]. *E. helvum* feed on fruits and blossoms
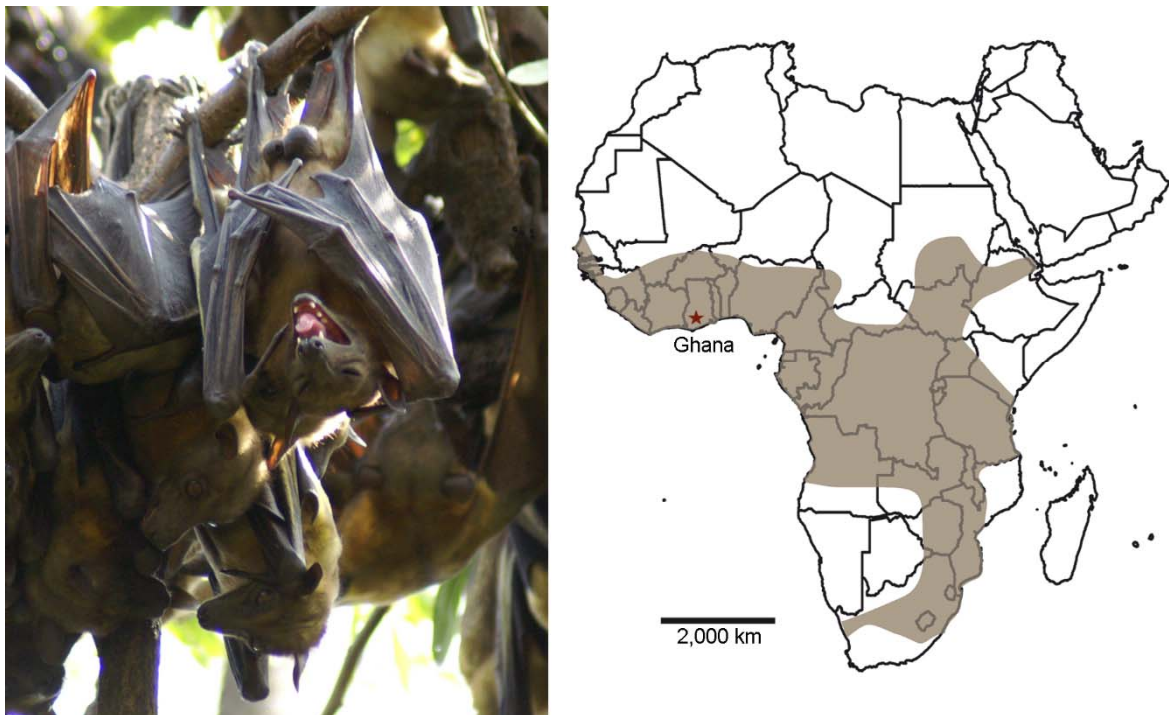
Figure 1: *E. helvum* **in the zoological garden of Kumasi and the habitat range of** *E.* *helvum*. This species exist on the African continent only, and migrates over long distances crossing country borders. The colony, studied in this thesis, resides temporally in Kumasi (red star), Ghana. Foto: F.Gloza-Rausch. Map modified according to [24].

and migration coincide with blossoming and fruiting of specific tree species [23]. During migration, colonies arrive at roosting areas when fruit abundance is increasing and continue to migrate when fruit abundance is decreasing, following the seasonal abundance of local food resources [22, 23]. As a result of deforestation and the expansion of human settlements, *E. helvum* are increasingly roosting in urban areas getting in closer contact with humans [25, 26]. Fruit bats have long lifespans and low rates of reproduction. Mating occurs seasonally in April to July but gestation does not begin until October. Females typically give birth in maternity colonies to one pup (occasionally two) in February to late-March prior to the onset of rainfall season [18, 27-29]. Increased use of urban habitats often creates conflicts with humans. Residents complain about noise and odour annoyance and depredation of crops. Hence *E. helvum* is often hunted, but not only for reasons of nuisance but also as a source of protein and income, if not used for self-consumption. In fact, *E. helvum* is one of the most hunted bushmeat in Sub-Saharan Africa. In Ghana, a minimum of 128,000 *E. helvum* bats are sold annually [26]. This is a serious concern, as fruit bats are essential for seed dispersal, pollination and the genetic connectivity of plants among fragmented patches of rainforest [22]. The resulting products of timber, fruit, fibres and tannins contribute significant to world markets and local economies [22].

1.2.1. Viruses in *E. helvum*

There is increasing evidence that *E. helvum* harbour a variety of viruses from different families. The first virus isolate from *E. helvum* was Lagos bat virus (LBV) from the genus *Lyssavirus* [30]. Later, antibodies against LBV were detected in colonies from Ghana [31, 32], Kenya [33] and Nigeria [34]. Antibodies against other members of the genus *Lyssavirus*, Rabies virus (Nigeria) and Mokala virus (Kenya, Ghana), were also detected [31, 33, 35]. In 2013, two related *Rubulaviruses* (Achimota 1 and 2) from the family *Paramyxoviridae* were isolated from a straw-coloured fruit bat in Ghana. The viruses are distantly related to the human pathogenic Mumps and Parainfluenza virus 2 and 4. Serum of *E. helvum* from Ghana and the islands São Tomé, Principe and Annobón contained neutralizing antibodies against the two novel *Rubulaviruses* [36]. At least 20 other previously unknown *Rubulaviruses* circulate in *E. helvum* colonies

across Sub-Saharan Africa [16]. *Henipaviruses* have not yet been isolated from *E. helvum*, but there is evidence of a high diversity of henipa viruses in these animals [16, 37], and serological cross-reaction and neutralization with Nipah virus and Hendra virus were observed [16, 38, 39]. Apart from an *Orbivirus* (family *Reoviridae*), which was isolated from a Nigerian straw-coloured fruit bat, there have been no other virus isolate from *E. helvum* until now [40]. However, metagenomic analysis's suggest the presence of viruses from the families *Reoviridae*, *Parvoviridae*, *Herpesviridae*, *Papillomaviridae*, *Adenoviridae*, *Poxviridae* and *Picronaviridae* [41-43]. It is therefore likely that increased research effort will uncover higher diversity of viruses hosted by *E. helvum*.

1.2.2. *E. helvum* colony in Kumasi

This study was conducted in a colony of approximately 300,000 individuals which roosts temporally in Kumasi, Ghana. Their primary roosting side is the zoological garden of Kumasi, located in central Kumasi, next to Kejetia market, the largest market in Western Africa. "Animals were first observed in July 1992. In March 1993 individuals were recorded in a coconut tree and spread within four weeks on more trees. In the following years, their number increased and roosting areas on prior neglected trees were occupied. Since 1995, almost all trees in the zoological garden of Kumasi were used as roosting areas" (pers. comm.). A second known roosting area, is the Botanical garden on the campus of the Kwame Nkruma University, at the outskirts of Kumasi. The colony visits Kumasi during its annual migration, typically arriving in October with increasing numbers until December. Although the colony size may fluctuate on a daily basis following available food resources, the roosting sites are occupied until at least April. Parturition occurs in March, but a small population of animals forms a resident population year-round. The colony has close contact with humans, being within the zoological garden and in close proximity to Kejetia market, and also on the university campus. Humans are exposed to urine and faeces of the bats, particularly workers of the zoological garden who both live and work there. Additionally, the animals are hunted for consumption and control reasons.

1.3. *Paramyxoviridae*

Paramyxoviruses are enveloped, negative-sense single strand RNA viruses that are divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. The subfamily *Paramyxovirinae*, comprises five genera, namely *Respirovirus*, *Rubulavirus*, *Morbillivirus*, *Henipavirus* and *Avulavirus*. Prominent human pathogens within this subfamily, are Human respiratory syncytial virus (genus *Pneumovirus*), Measles virus (genus *Morbillivirus*) and Mumps virus (genus *Rubulavirus*). Viruses in the subfamily *Paramyxovirinae*, have been associated with a number of emerging diseases in humans and animals, in the past two decades [61-68]. In 1994, a novel paramyxovirus named Hendra virus, associated with respiratory disease in horses and humans, caused two outbreaks in Australia [69, 70]. In the second outbreak, a patient with contact to horses, that had died of severe respiratory syndrome, died from relapsing encephalitis [71, 72]. Hendra virus continues to cause re-emerging outbreaks in Australia. Nipah virus, is another novel *Paramyxovirus*, which emerged in Malaysia in 1998, causing an outbreak of febrile encephalitis among pig farmers. The outbreak was linked, later, to cases of respiratory and neurological disease in domestic pigs [73, 74]. Since then, Nipah virus has caused several outbreaks in Malaysia, Singapore, India and Bangladesh causing lethal outcomes in many cases. These two viruses were assigned to a novel genus, *Henipavirus*, within the *Paramyxovirinae* [75]. Until now, they are the only assigned viruses in this genus. Although, human cases have been linked to contacts with horses and pigs, *Pteropus* bats, commonly known as flying-foxes, are suggested as wildlife reservoir for both viruses [64, 76]. Evidence of *Henipavirus* infection, in flying-foxes of different species, was found in China [77], Thailand [78, 79], Cambodia [80], Papua New Guinea [81], Madagascar [82] and Ghana [37, 39]. In Ghana, antibodies against henipaviruses were detected in domestic pigs [83]. However, none of the afore mentioned countries have reported *Henipavirus* outbreaks. The transmission route of henipaviruses is hypothesized to be *via* urine and saliva. Outbreaks are associated with *Pteropus* bats roosting in close proximity to horses and piggeries. The viruses are transmitted via droppings or contaminated fruits, to horses and pigs in which they are amplified and further transmitted to humans [84-86]. In Bangladesh, *Pteropus* bats feed on date palm sap and transmission of Nipah virus to

humans, consuming contaminated date palm sap, occurred [87]. The only cases of human-to-human transmission were reported from Bangladesh [88, 89]. Until now, no cases of direct bat to human transmission of henipaviruses are known. Recently, Cedar virus, a virus related to Hendra- and Nipah virus was isolated from an Australian fruit bat [90]. Antibodies to Cedar virus cross-react with Hendra and Nipah virus, but cross-neutralization was not observed. In experiments with ferrets and guinea pigs, which are susceptible to Hendra and Nipah virus, no clinical signs developed [90]. In the genus *Rubulavirus* seven novel, with fruit bat associated viruses, were detected in the recent years. Menangle virus was originally isolated from stillborn piglets in Australia [61]. The virus circulated briefly in piggeries before it was eradicated in 1999 [91]. However, two infected humans developed severe influenza-like illness and rash [92]. Neutralizing antibodies were detected in Australian *Pteropus*
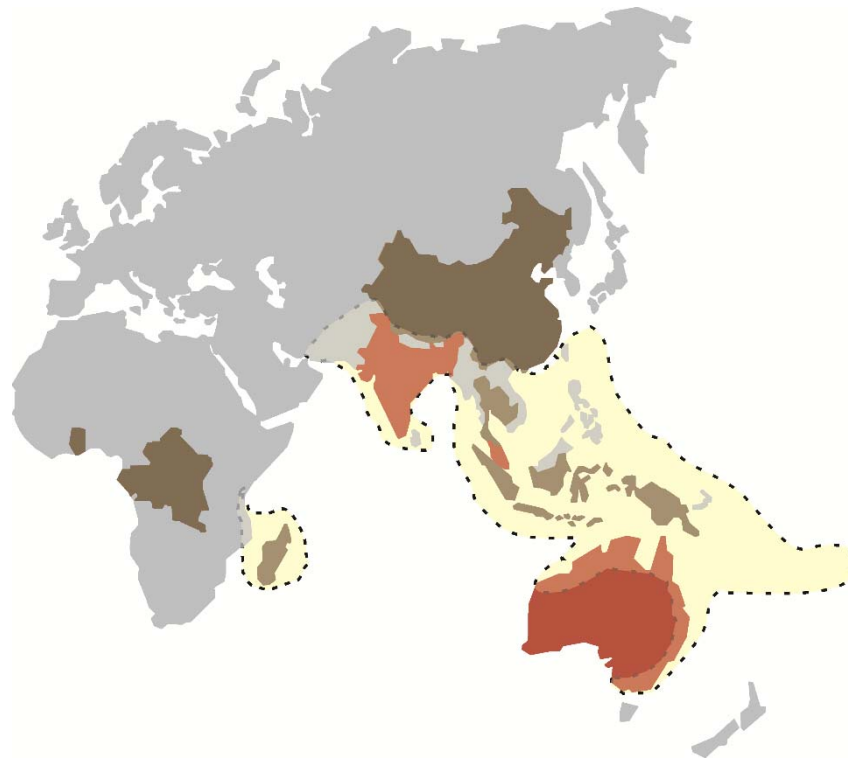
Figure 3: **Global distribution of *Henipaviruses*.** Outbreaks of, Hendra- or Nipahvirus, were reported from Australia, Malaysia, Singapore, Bangladesh and India (red). Serological evidence and/or viral RNA of henipaviruses, in flying foxes, were detected in South-East Asia but also in Africa (brown). The distribution of *Pteropus* bats is shaded in yellow. Picture modified according to [93].

bats and the virus was isolated from bats in 2012, linking Menangle virus to fruit bats [91, 94, 95]. In Ghana, two *Rubulaviruses*, Achimota 1 and 2, were isolated from fruit bats [36]. Achimota virus 1 and 2 neutralizing antibodies, were detected in several fruit bat colonies across Sub-Saharan Africa. Although neutralizing antibodies were detected in humans, no link to a disease was made [36]. Tioman virus, was isolated from a fruit bat of Tioman island, a small island off the east coast of Malaysia [96], and neutralizing antibodies in *Pteropus* were also detected in Madagascar [82, 96]. Tuhoko virus 1-3 from China, related to Menangle- and Tioman virus, have not yet been isolated but antibodies have been detected in Leschenault's rousette bats [97]. None of the mentioned viruses caused clinical signs of illness is bats. In humans, only infection with Hendra-, Nipah- or Menangle virus lead to the development of a disease. In the past, the detection and characterisation of novel viruses on the base of genetic information, was impossible. However, the development of deep sequencing and enhanced tools for molecular biology, are expected to lead to a rapidly increase in the detection of novel viruses.

1.4. *Rhabdoviridae*

The family *Rhabdoviridae* contains >250 known rhabdoviruses, currently classified in six acknowledged genera (*Lyssaviruses*, *Vesiculovirus*, *Ephemerovirus*, *Novirhabdovirus*, *Nucleorhabdovirus* and *Cytorhabdovirus*). According to the International Committee on Taxonomy of Viruses (ICTV), three more genera are currently pending (*Perhabdovirus*, *Sigmavirus* and *Tibrovirus*) and >100 rhabdoviruses are still unclassified [44]. *Rhabdoviridae* are enveloped viruses, with a negative-sense single-stranded RNA and a typical bullet shape virion. The general genome structure is nucleocapsid (N) - phosphoprotein (P) - matrixprotein (M) - glycoprotein (G) - large protein (L), however a variety of rhabdoviruses contain genes between P - M, M - G and/or G - L. The complexity of the genome is increased with overlapping reading frames (ORF) within genes (e.g. P and G) or in novel ORFs, for some species [45]. All plant rhabdoviruses
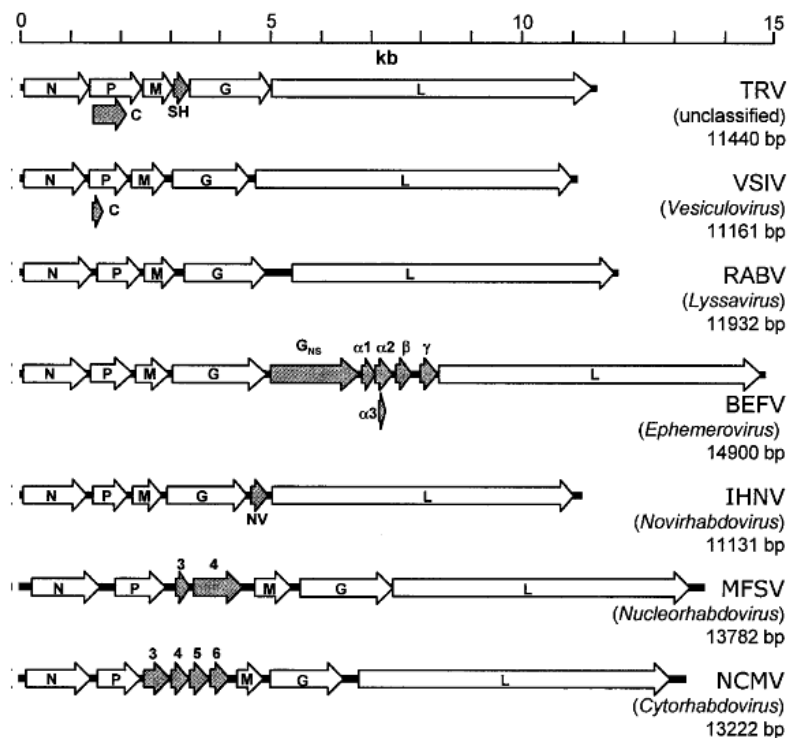
Figure 2: **Comparison of the genome structure of representatives of different rhabdovirus genera.** The reading frames for the conserved rhabdovirus genes N, P, M, G and L are depicted as open arrows, additional genes are shown in grey. The size of the genomes and the rhabdovirus genera are indicated. According to [46].

(*Nucleorhabdovirus* and *Cytorhabdovirus*) typically encode more than the usual five genes. At least one, and a maximum of four genes, are inserted between the P and M gene [47, 48]. Fish rhabdoviruses (some *Vesiculorhabdoviruses* and *Novirhabdoviruses*) have an additional gene between G and L. *Ephemeroviruses* encode additional genes between G and L [48]. Representatives of different rhabdovirus genera are shown in (Fig. 2). Universal phylogenetic trees of the *Rhabdoviridae*, are traditionally generated by using sequences of the N gene [49]. The degree of conservation decreases in the order N > L > M > G > P [47]. Each of the five individual genes is flanked by transcription initiation and termination/polyadenylation signals, which may be conserved among members of the same genus [47]. Between each transcription unit (gene and associated flanking signals) is a nontranscribed intergenic region that usually contains a single or dinucleotide sequence [e.g. G or GG in Tupaia rhabdovirus (TUPV)] [45]. Termini of rhabdoviruses are highly conserved with an inverse complementary sequence of 15-20 nt, rich in A/U content, at both ends. These regions contain the genomic and antigenomic promoters, essential for viral replication and transcription [50]. In mammalian rhabdoviruses, the terminal nucleotides are conserved as 5'-ACG/CGT-3' [48, 50]. Rhabdoviruses have been shown to infect all organisms, except bacteria (mammals, reptiles, fish, insects, fungi, and plants), however, they are rarely associated with diseases in humans [51]. The majority have two natural hosts: either insect and plants or insects and vertebrates, although never all three [47]. Five of the six rhabdovirus genera contain viruses that are transmitted and/or hosted by insects. Only fish rhabdoviruses and *Lyssaviruses* are not maintained by insect hosts. It is therefore postulated that *Rhabdoviridae* evolved from an ancestral insect virus. The supergroup dimarhabdovirus (dipteran-mammal associated rhabdoviruses) summarise arthropod-transmitted animal rhabdoviruses. It comprises the genera *Ephemero-* and *Vesiculovirus* and a variety of unassigned rhabdoviruses. Included in this group are the viruses Bovine ephemeral fever virus (BEFV) [52], Kontonkan virus (KOTV) [53] and Vesiculo Stomatitis virus (VSV) [52-54] which cause severe disease in cattle. With the exception of Rabies virus, rhabdoviruses are generally not associated with diseases in humans. However, three viruses from the dimarhabdo supergroup cause fatal disease in humans. Chandipura virus (CHPV), has caused outbreaks of encephalitis in India, and has also been detected in Africa [55]. Le

Dantec virus [56] and the recently described Bas-Congo virus (BASV) [57], have caused individual cases of hemorrhagic fever in Africa. Three dimarhabdoviruses have been isolated from bats: Oita virus (OIRV) [58], Mount Elgon bat virus (MEBV) [59] which both originate from Kenya, and Kern Canyon (KCV) which was isolated from a North American bat [59]. These viruses form a monophyletic clade and are probably geographic variants, which are common for rhabdoviruses. In the genus *Ephemerovirus*, the Australian viruses Kimberley- and Adelaide river virus are probably geographic variants of the African Malakal- and Obodhiang virus [60]. So far, the role of bats in the evolution and transmission of rhabdoviruses is still unclear.

1.5. Aim of the thesis

The focus on bats as reservoirs of potentially emerging diseases has increased in the last decades. Most studies focus on the detection of viruses without exploring their genetic diversity to lower taxonomic levels, for example, to genera and species within bat colonies. Even less is known about the ecology and transmission patterns of these viruses.

The aim of this thesis is to investigate bat virus diversity and dynamics in a longitudinal approach. The 300,000 strong colony of *E. helvum* in highly populated Kumasi, Ghana, provides a study site where bat-human interaction occurs on a daily basis. The potential for zoonotic transmission is thus potentially high. Previous studies have shown a high diversity of *Paramyxoviridae* genera *Henipa-* and *Rubulavirus* in fruit bats. Therefore, investigation of the virus diversity in the *E. helvum* colony focused on these genera.

For the study, an *E. helvum* organ collection was generated over a time frame of three years. *E. helvum* organs were screened for the presence of novel and known *Paramyxoviridae*, and virus sequences were compared to their abundance during the sampling time, their relation to other fruit bat viruses and distribution in different African countries.

I aimed to isolate viruses from *E. helvum* and characterise virus abundance in the colony. Possible transmission pathways were investigated by testing for organ tropism. For isolated viruses, serological assays were established to define the serological status of the *E. helvum* colony and investigate potential cross-species transmission of bat viruses to livestock and humans.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

100 bp DNA ladder	Life Technologies, Darmstadt, Germany
2-Mercaptoethanol (β-Mercaptoethanol)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
ACCUGEN, RNAse free water	Lonza Cologne, Cologne, Germany
Acetic acid, 100%, Ph.Eur., reinst	Carl Roth GmbH + Co. KG, Karlsruhe
Agarose Broad Range	Carl Roth GmbH + Co. KG, Karlsruhe
Agarose GTQ	Carl Roth GmbH + Co. KG, Karlsruhe
Ampuwa® (sterile, pyrogen-free water)	Fresenius Kabi, Bad Homburg, Germany
Beta propiolacton	Ferak Berlin, Berlin, Germany
Bovine Serum Albumin (BSA)	New England Biolabs GmbH, Frankfurt, Germany
Bovine Serum Albumin	Roche Diagnostics, Mannheim, Germany
Bromphenol blue	Sigma-Aldrich Chemie GmbH, Munich, Germany
Carrier RNA (10 mg/mL)	QIAGEN, Hilden, Germany
Chloric acid (HCl)	Carl Roth GmbH + Co. KG, Karlsruhe
Coomasie Plus^{TM} (Bradford solution)	Thermo Scientific, Bonn, Germany
Crystal Violet	Carl Roth GmbH + Co. KG, Karlsruhe
DAPI ProLong Gold antifade reagent	Invitrogen, Karlsruhe, Germany
Disodium hydrogen phosphate – dihydrate (Na_2HPO_4-$7H_2O$)	Merck KGaA, Darmstadt, Germany
dNTP set (dATP, dTTP, dGTP, dCTP)	Invitrogen, Karlsruhe
Ethanol ≥99.9%	Carl Roth GmbH + Co. KG, Karlsruhe
Ethidium Bromide (10 mg/mL)	Carl Roth GmbH + Co. KG, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	AppliChem, Darmstadt, Germany
EUROIMMUN sample buffer	EUROIMMUN AG, Lübeck. Germany
Formaldehyde 37%	Carl Roth GmbH + Co. KG, Karlsruhe
Glycerol	Carl Roth GmbH + Co. KG, Karlsruhe
Ketamin 10%	Medistar, Ascheberg, Germany
LB-Agar (Lennox)	Carl Roth GmbH + Co. KG, Karlsruhe
Magnesium chloride (PCR)	Invitrogen, Karsruhe
Methanol (99%)	Carl Roth GmbH + Co. KG, Karlsruhe
Milk powder	Carl Roth GmbH + Co. KG, Karlsruhe
Natriumhydrogencarbonat	Carl Roth GmbH + Co. KG, Karlsruhe
Roti®-Histofix 4% (pH7)	Carl Roth GmbH + Co. KG, Karlsruhe
Sacharose	Sigma-Aldrich Chemie GmbH, Munich
Sodium hydroxide (NaOH)	Carl Roth GmbH + Co. KG, Karlsruhe
Tris hydroxymethyl aminomethane (Tris)	Carl Roth GmbH + Co. KG, Karlsruhe
Triton X-100	Sigma-Aldrich Chemie GmbH, Munich,
Tween 20	Sigma-Aldrich Chemie GmbH, Munich
Xylene cyanol FF	Sigma-Aldrich Chemie GmbH, Munich
Xyxlazin (Rompun®)	Bayer, Leverkusen, Germany

2.1.2. Buffers and Solutions

Name	Ingredients
6x Loading Dye	40g Sacharose
	0.25 g Bromphenol blue
	0.223 g EDTA
	in 100 mL deionized water
Crystal violet stock solution	10 g Crystal violet
	50 mL Formaldehyde (37%)
	100 mL Ethanol (99.9%)
	350 mL deionized water
Crystal violet working solution	100 mL Crystal violet stock solution
	100 mL Formaldehyde (37%)
	800 mL deionized water
PBS-Tween	0.1% Tween$_R$ 20
	10% 10x PBS
	in deionized water
Phosphate buffered saline (PBS) 10x, pH7.0	80 g NaCl
	2 g KCl
	26.8 g Na_2HPO_4-$7H_2O$
	2.4 g KH_2PO_4
	adjust pH with 37% HCl
	add 1 L deionized water
	autoclave
TBE 10x	121 g Tris
	61.8 g boric acid
	186.12 g EDTA
	in 1L deionized water

2.1.3. Consumables

12-well immunoslides 5mm	Dunn Labortechnik GmbH, Asbach, Germany
C-Chip, Disposable Neubauer improved counting chamber	Biochrom AG, Berlin, Germany
Cell culture flask with filter cap (25, 75, 175 cm2)	SARSTEDT AG & Co., Numbrecht, Germany
Cell culture plate (48-well)	SARSTEDT AG & Co., Numbrecht
Cell culture plates (6-well, 24-well)	SARSTEDT AG & Co., Numbrecht
Cell scraper	TPP Techno Plastic Products AG, Trasadingen, Switzerland
Centrifuge tubes (15, 50 mL)	SARSTEDT AG & Co., Numbrecht
Cryotubes	SARSTEDT AG & Co., Numbrecht
LightCyclerR Capillaries (20 XL)	Roche Diagnostics GmbH, Mannheim, Germany
LightCyclerR480 Multiwell Plate 96, white	Roche Diagnostics GmbH,Mannheim
Master point Energie Cal 4,5 (.177)	Industrias el Gamo, Barcelona, Spain
Needles 21G	Servopax GmbH, Wesel, Germany
Nunc Maxi Sorp 96-well plates	Thermo Fisher Scientific, Schwerte, Germany
PCR reaction tubes (0.2 XL)	SARSTEDT AG & Co., Numbrecht
Pipette Tips (10, 20, 200, 1000 XL)	SARSTEDT AG & Co., Numbrecht
Reaction tubes (1.5, 2 mL)	SARSTEDT AG & Co., Numbrecht
Scalpel (No 15, 11)	Feather Safety Razor Co., Osaka, Japan
Serological pipettes (1, 2, 5, 10, 25 mL)	SARSTEDT AG & Co., Numbrecht
S-Monovette EDTA K_2 (10 mL)	SARSTEDT AG & Co., Numbrecht
Stericup and Steritop Vacuum Filter Cups (500 mL)	Millipore GmbH, Schwalbach, Germany
Syringe (1, 2, 5 mL)	BD, Heidelberg, Germany
Syringe Filter (0.2 µm)	Pall Corporation, Ann Aror, USA
UlltraClear tubes (15 mL, 50 mL)	Beckman Coulter, Krefeld, Germany

2.1.4. Technical Equipment

Equipment	Type	Source
454 sequencer	GS Junior	Roche Diagnostics GmbH,Mannheim
Air rifle	Diana Panther 21	Mayer & Gummelsbacher GmbH, Rastatt, Germany
Autoclave	V120	Systec GmbH, Wettenberg, Germany
Balance	SPO 61	Scaltec Instruments GmbH, Göttingen, Germany
Centrifuges	Centrifuge 5424	Eppendorf, Hamburg, Germany
	Centrifuge 5810R	Eppendorf, Hamburg
	Sorvall Evolution RC	Thermo Fisher Scientific, Schwerte
Chemiluminescence reader	SynergyTM 2	BioTek, Bad Friedrichshall
	Spectramax 190	Molecular Device, Sunnyvale, USA
Dryshipper	MVE SC 20/12 V SC 4/2 XC 20/3 V	German-cryo®GmbH, Jülich Germany
Freezer	-20°C Liebherr premium	Liebherr, Biberbach a. d. Ris, Germany
	-80°C/Typ499	Kaltis Europe GmbH, Niederweningen, Switzerland
	Liquid Nitrogen LS 750	Taylor Wharton Germany GmbH,Husum
Gel electrophoresis	PerfectBlue Gelsystem MaxiS 200 mL	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Gel electrophoresis documentation	E-Box 3028, WL/26M	Vilbert Lourmat, Marne-la-Vallee, France
Heating block	Thermomixer comfort	Eppendorf, Hamburg
Hood (Bioflow)	HeraSafe	Thermo Fisher Scientific, Schwerte
Incubators	HERAcellR 240	Thermo Fisher Scientific, St. Leon-Roth, Germany
	HeraeusR B6126	Thermo Fisher Scientific, St. Leon-Roth
Microscopes	TELAVAL31	Carl Zeiss GmbH, Jena, Germany
	IMAGER.M1	Carl Zeiss GmbH, Jena
PCR cycler	Mastercycler epgradient S	Eppendorf, Hamburg
pH meter	766 Calimatic	Knick Elektronische Messgeräte

		GmbH & Co. KG, Berlin, Germany
Photometer	NanoDrop 2000c	PEQLAB Biotechnologie GmbH, Erlangen
	Biophotometer	Eppendorf, Hamburg
Pipette assistance	Accu-jetR pro	Brand, Wertheim, Germany
Pipettes	Research, PhysioCare (100-1000 μL, 20-200 μL,2-20 μL, 0.5-10 μL)	Eppendorf, Hamburg
Power supply	EV202	Consort, Turnhout, Belgium
Real-time PCR cycler	LightCyclerR 1.5	Roche Diagnostics GmbH, Mannheim
	LightCyclerR 480	Roche Diagnostics GmbH, Mannheim
Rocking Block	Mini Rocker MR.1	PEQLAB Biotechnologie GmbH, Erlangen
Rotor	SW40 Ti, SW41 Ti	Beckman Coulter, Krefeld, Germany
Tissue Lyser	Qiagen	Retsch Inc., Newtown, USA
Ultrazentrifuge	Optima L-80 XP	Beckman Coulter, Krefeld
Vortexer	Vortex VF2	IKAR-Werke GmbH & CO. KG, Staufen, Germany
Water purification system	Milli-QR Biocel	Millipore GmbH, Schwalbach, Germany

2.1.5. Cell culture media and supplements

Amino Acids Non Essential (100x, 50 mL)	PAA Laboratories GmbH, Cölbe
Amphotericin B (250μg/mL)	PAA Laboratories GmbH, Cölbe
Avicel RC581	FCM BioPolymer, Brussels, Belgium
CryoMaxx S (50 mL)	PAA Laboratories GmbH, Cölbe
Dulbecco's Modified Eagles Medium (high glucose, 4.5 g/L, 500 mL) (DMEM)	PAA Laboratories GmbH, Cölbe
Dulbecco's PBS without Mg/Ca(1x, 500 mL)	PAA Laboratories GmbH, Cölbe
Earl MEM (9.69 g/L)	Biochrom AG, Berlin, Germany
Fetal Calf Serum (FCS) "Standard" (100 mL)	PAA Laboratories GmbH, Cölbe
Imipinem/Cilastin (Zienam ®) (500 mg)	MSD Sharp&Dohme GmbH, Haar, Germany
L-glutamine (20 mM, 50 mL)	PAA Laboratories GmbH, Cölbe
OptiPRO™ serum-free medium (1 L)	Life Technologies, Darmstadt, Germany
Penicillin/Streptomycin (100x, 50 mL)	PAA Laboratories GmbH, Cölbe
Sodium pyruvat (100 mM, 50mL)	PAA Laboratories GmbH, Cölbe
Trypsin EDTA (1x, 50 mL)	PAA Laboratories GmbH, Cölbe

2.1.6. Cell lines

Name	Source
Vero E6	Monkey kidney cell line (ATCC® CRL-1586)
Vero FM	Monkey kidney cell line (kind gift of Jindrich Cinatl, Universtiy of Frankfurt)
MA104	Monkey kidney cell line (cell culture collection Bernhard Nocht-Institute for Tropical Medicine, Hamburg)
A549	Human lung carcinoma cells (ATCC®CCL-185)
EidNi	Eidolon helvum kidney cell line (home made)
EidLu	Eidlon helvum lung cell line (home made)

2.1.7. Antibodies

Donkey-anti-goat Cy2	Dianova, Hamburg, Germany
Donkey-anti-sheep Alexa Fluor488	Dianova, Hamburg
Goat-anti-bat antibody IgG	Bethyl Laboratories, Montgomery, USA
Goat-anti-bovine Alexa Fluor488	Dianova, Hamburg
Goat-anti-human Cy2	Dianova, Hamburg
Goat-anti-swine Alexa Fluor488	Dianova, Hamburg
Goat-α-bat-HRP	Dianova, Hamburg
Goat-α-human-HRP	Bethyl Laboratories, Montgomery

2.1.8. Oligonucleotides

Hemi-nested reverse transcription (RT) PCR

Paramyxoviridae

RES-MOR-HEN-F1	TCI TTC TTT AGA ACI TTY GGN CAY CC
RES-MOR-HEN-F2	GCC ATA TTT TGT GGA ATA ATH ATH AAY GG
RES-MOR-HEN-R	CTC ATT TTG TAI GTC ATY TTN GCR AA
AVU-RUB-F1	GGT TAT CCT CAT TTI TTY GAR TGG ATH CA
AVU-RUB-F2	ACA CTC TAT GTI GGI GAI CCN TTY AAY CC
AVU-RUB-R	GCA ATT GCT TGA TTI TCI CCY TGN AC
PNE-F1	GTG TAG GTA GIA TGT TYG CNA TGC ARC C
PNE-F2	ACT GAT CTI AGY AAR TTY AAY CAR GC
PNE-R	GTC CCA CAA ITT TTG RCA CCA NCC YTC

Colony PCR

M13mod-F	GTAAAACGACGGCCAGTGAAT
M13mod-R	CACACAGGAAACAGCTATGAC

Real time RT PCR

Kumasi rhabdovirus

BtRhabdoM17-rt F	CTGACTATCGCGACATGCTGTAC
BtRhabdoM17-rtP	FAM[a]-ACACGGCGAAAGATCATGCCAAACA-BHQ1[b]
BtRhabdoM17-rt R	TCCATTGCTCTCTGGCTCAA

Henipa-like viruses

Spl6RMH-F	CGGGATAGACATGGAGGTGTGT
Spl3+6RMH-P	FAM-CCITCTTGTTTCCTTCCTGATCATGCATC-BHQ1
Spl6RMH-R	CCGTTCATCTTTTTGGATTTGAT
Spl3RMH-F	CGAGATAGACATGGAGGTGTATG
Spl3+6RMH-P	FAM-CCITCTTGTTTCCTTCCTGATCATGCATC-BHQ1
Spl3RMH-R	TTCTGCGCAATCCTCTATTGTCA
Spl2RMH-F	TTTACCCTTCCATCAACCTACGTT
Spl2RMH-P	FAM-CAACCCTCCTCAATCGTCCACTTCCA-BHQ1
Spl2RMH-R	TCTGTGTCCTTTAGATATTCTCCTGATATT
Spl33nRMH1-F	TGGTGTCTGGCCTCCTATGAA
Spl33nRMH1-P	FAM-TTCCCCAGGCATGTTTCAAATACCATCA-BBQ[c]
Spl33nRMH1-R	CATATGTAAGTCTGTCTCCAGATGATTG
Spl28nRMH2-F	AGATAGACACGGAGGGATTTGG
Spl28nRMH2-P	FAM-TGCAAACTTCCAGATCATTGTTCACCTCA-BBQ
Spl28nRMH2-R	TCTCCGTTCATTTTTTTGCTTTT
PVSpl43RMH-F	TTGTGGCACCATAATAAATGGATT
PVSpl43RMH-P	FAM-ACTTGGCCTCCTTGCGAACTTCCTG-BHQ1
PVSpl43RMH-R	CTCTTAACCAGAGCAGAAGCATGA
PV-Spl90-69RMH-F	GTTCAGAGACAGACATGGAGGTATGT
PV-Spl90-69RMH-P	FAM-TGTGACCTCCCTCCACATTCTTCACCTC-BHQ1
PV-Spl90-69RMH-R	TGGATAAGGACTCAGCATTAAGTTGT
PV-Spl67-51RMH-F	TTTGTGGGACAATTATCAATGGAT

PV-Spl67-51RMH-P	FAM-TGGCACCTGGCCACCATGTTCTCT-BHQ1
PV-Spl67-51RMH-R	TTTTTATAAGAGGTGAAGCATGATGTG
PV-Spl48-55-91-27a-F	AAGCTTTGTCTCCCATTAAATCACA
PV-Spl48-55-91-27a-P	FAM-AATGCCAACATGAAATACACACCAAAGCCT-BHQ1
PV-Spl48-55-91-27a-R	GGTTCAAACTCAGCATCATTGATAA
PV-Spl63-65-F	CAGTCCTTAATTGGCGTTCGTT
PV-Spl63-65-P	FAM-ATTGGGTTCAAATTTGGTGTAT-MGBNFQ[d]
PV-Spl63-65-R	TCACTATCGAGGTCTAAGGGCATA
PV-F7-F	AAAGCTCTTGCTGCGATAAGGA
PV-F7-P	FAM-TGAGTGGGACAGTGTTT-MGBNFQ
PV-F7-R	GTTGGCTGGTAAGAGAGATTTTCC

aFAM, 6-carboxyfluorescein; bBHQ1, Black Hole Quencher1, cBBQ, Black Berry Quencher, dMGB, Minor Groove Binder; NFQ, Non-fluorescent Quencher, R=G/A, Y=C/T, S=G/C, W=A/T, H=A/C/T, N=any base, I=inosine

2.1.9. Enzymes

Platinum®Taq DNA Polymerase	Invitrogen, Karlsruhe
SuperScript™ III One-Step RT-PCR System with Platinum Taq DNA Polymerase	Invitrogen, Karsruhe

2.1.10. Kits

cDNA Synthesis Kit	Roche Diagnostics GmbH, Mannheim
GeneRacer Kit	LifeTech, Carlsbad, USA
GS FLX Titanium Rapid Library Preparation Kit	Roche Diagnostics GmbH, Mannheim
MegaScript T7® Kit	Invitrogen, Karlsruhe
NucleoSpin® RNA II	Macherey-Nagel, Düren, Germany
NucleoSpin® RNA virus	Macherey-Nagel, Düren
QIAamp MinElute Virus Spin	QIAGEN, Hilden
QIAamp RNeasy® Mini Kit	QIAGEN, Hilden
QIAamp Viral RNA Mini Kit	QIAGEN, Hilden
QIAprep Spin Miniprep-Kit	QIAGEN, Hilden
SeqLab	Sequence Laboratories, Göttingen, Germany
TOPO® TA Cloning® kit	Invitrogen, Karlsruhe

2.1.11. Software

Adobe Photoshop CS4
AxioVision Rel. 4.8
BEAST V1.7.4
BioEdit Sequence Alignment Editor version 7.0.5.3
Coral DRAW® X3
DNASTAR Lasergene 7 (EditSeq, SeqMan)
EpiInfo V7
Gen5
Geneious 5.1
MAFFT V7
MEGA 5.2
MrBayes V.3.1
Newbler software (Roche)
SOFT max Pro 3.0
IBM SPSS Statistics 22.

2.2. Methods

2.2.1. Field sampling

For all capturing and sampling, permission was obtained from the Wildlife Division, Forestry Commission, Accra, Ghana. Ethical approval for human samples was provided by the Committee on Human Research, Publications and Ethics Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Samples were exported under a state contract between the Republic of Ghana and the Federal Republic of Germany, and export permission from the Veterinary Services of the Ghana Ministry of Food and Agriculture. All samples were transported in liquid nitrogen. Between 2009 and 2012, the *E. helvum* colony in Kumasi was visited once a week in the months October to April. On average twelve animals were sacrificed per week. Identification and capturing was done by trained field biologist. Animals were shot from trees with an air rifle, anesthetised with a Xylazin:Ketamin (1:4) mixture and blood was taken by heart puncture. The carcasses were transported on ice to the close by Kumasi Centre for Collaborative Research were spleen, liver, kidney, intestine, gut, lung and brain were dissected. Aliquots of each organ were directly snap-frozen and preserved in 4% formaldehyde. Urine samples were collected in June 2011 by placing a clean plastic sheet under a tree visited by *E. helvum*. Droppings were collected directly and stored at 4°C for transport and frozen at -80°C. In July 2011, blood samples were taken by trained phlebotomists from people working at the Zoological gardens of Kumasi after obtaining informed consent from all participants. Livestock samples were taken in December 2011 by trained veterinarians. Generally, blood was drawn from the vena jugularis externa. Animals originated from Kumasi and surrounding areas. All sera were collected in EDTA Monovettes. Samples were stored at 4°C for several hours before sepearation by centrifugation at 2,500 x g for 10 minutes and aliquoted and stored at -80°C.

2.2.2. Cell culture methods, virus isolation and propagation

2.2.2.1. General cell culture methods

Cell lines were maintained in DMEM supplemented with 10% fetal-calf serum (FCS), 1% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids and 1% L-glutamine (hereafter referred to as supplemented DMEM) at 37°C, 5% CO_2. According to cell growth, cells were passaged one to three times a week. Supernatant was removed, cells were washed with PBS and incubated with trypsin at 37°C until all cells detached from the surface. Trypsin was inactivated by resuspending cells in culture medium and cells split in ratios between 1:3 and 1:10 depending on cell growth.

2.2.2.2. Virus isolation

For the isolation of viruses from *E. helvum*, two heterogeneous cell lines derived from the lungs and kidneys of *E. helvum* were applied. In addition, VeroE6 cells were used as many viruses were previously isolated on this cell line (Lassavirus, Measles virus, Ebola virus, Crimean-Congo hemorrhagic fever virus, SARS virus) [98]. Body temperature of fruit bats are in the range of 30°C to 40°C with an average of 34°C to 37°C [99]. However, the effect of the temperature on bat virus growth is not known. Hence the temperature was maintained at 37°C which is the normal temperature for mammalian cell lines. Bat viruses are thought to be highly adapted to their hosts. A frequently observed pattern for these kind of viruses is the persistence or long-term replication in lymphatic organs as the spleen [16]. Therefore, the spleen was the organ of choice for virus isolation experiments.

2.2.2.3. Undirected virus isolation

The spleen of ten *E. helvum* from the year 2010 were inoculated on a mixture of EidNi/EidLu (1:1) cells and VeroE6 cells. Spleen were homogenized with the back of a syringe, frozen and thawed on ice. 5 x 10^4 cells/ml were seeded in 24-well plates. The Spleen suspension was centrifuged for 10 minutes at 300 x g, cells washed with PBS and infected with 200 µL cleared spleen suspension. Inoculum was removed after

1h of incubation at 37°C, 5% CO_2, cells washed with PBS and incubated in 1 mL supplemented DMEM with 5% FCS. Cells were observed daily for formation of CPE. Samples were blind-passaged six days post infection with 200 μL supernatant to fresh cells if no CPE formed. After six passages without formation of CPE virus isolation was closed. CPE positive samples were passaged with 200 μL supernatant on fresh VeroE6 cells, with each passage the cell area was increased to increase virus titre and volume.

2.2.2.4. Directed Virus isolation

Directed virus isolation was approached as described in undirected virus isolation (2.2.2.3.) but spleen were pre-tested with a genus-specific PCR (2.2.6.1.) for *Paramyxoviridae*. 12 spleen positive for the genus *Henipavirus* were used as inocula for virus isolation. In addition, 12 *E. helvum* urine samples positive for the genera *Henipavirus*, *Avulavirus* and *Respirovirus* were used. Urine (10-50 μL) was diluted in 850 μL supplemented DMEM. VeroE6, EidNi and EidLu cells were infected with 200 μL urine/medium mixture. The remaining sample was filtered through a 0.2 μm pore filter and cells infected with 200 μL cleared sample. After incubation at 37°C, 5% CO_2 for 1h, 1mL supplemented DMEM with 5% was added and cells incubated at 37°C in 5% CO_2. Cells were observed daily for formation of CPE and blind passaged with 200 μL supernatant to fresh cells if no CPE showed. If no CPE formed after six passages cell cultures were discarded.

2.2.2.5. Production of virus stock

VeroFM cells were seeded in a T163 flask ($1x10^7$ cells), infected with a multiplicity of infection (MOI) of 0.01 in 10mL OptiPRO™ (serum free medium) and incubated for 1h at 37°C, 5% CO_2. 10 mL supplemented DMEM was added and cells incubated at 37°C, 5% CO_2 for two days. Supernatant was harvested, cells opened with one cycle of freeze-thawing and resuspended in the virus-containing supernatant. Virus stock was cleared by centrifugation at 300 x g for 10 minutes, aliquoted and stored at -80°C until required.

2.2.2.6. Concentration of viral particles

Virus was produced as described in 2.2.2.5. but directly used for ultracentrifugation by one of two available systems depending on volume. For small volumes, 2 mL 36% saccharose was overlaid with 16 mL supernatant and centrifuged in a SW40 Ti rotor at 28,000 x g for 4h at 4°C. Large volumes were centrifuged in a SW41 Ti rotor with 5 mL 36% saccharose and 20 mL supernatant at 28,000 x g for 4h at 4°C. After concentration, supernatant was removed and the pellet resuspended in 200 μL 1x PBS overnight. Depending on the requirements for further experiments, viruses were inactivated by incubation in 2% paraformaldehyde (Pfa) for 20 minutes, inactivation buffer of the respective kit or with 0.1% β-propiolacton at 4°C overnight followed by 2h at 37°C.

2.2.2.7. Purification of viral particles

For 454 sequencing it is necessary to reduce the cellular background as much as possible. Therefore, viral particles were cleared on a saccharose gradient. Concentrated viral particles (2.2.2.6.) were overlaid on a saccharose gradient ranging from 10% to 60% and ultracentrifuged in a SW40 Ti rotor at 28,000 x g for 12h at 4°C. Fractions of 500 μL each were collected; RNA was extracted from an aliquot of 70μL for each fraction (2.2.5.4.) and tested in real-time RT-PCR (2.2.6.2.) for viral concentration. Fractions with the highest RNA concentrations were pooled and concentrated according to 2.2.2.6. of viral particles.

2.2.2.8. Detection of viral particles in cell culture

Virus was prepared as described in 2.2.2.6. and inactivated in 2% Pfa. Inactivated virus was sent to Andreas Kurth at the Robert Koch Institute in Berlin, by whom electron microscopic analysis and image capture of viral particles was done.

2.2.2.9. Plaque titration assay

VeroFM cells were seeded with a concentration of 4×10^5 cells/mL (0.5 mL/well) in a 24-well plate and incubated over night. Cells were washed with 1x PBS. Virus stock was diluted from 10^{-1} to 10^{-6} in OptiPRO™. For each dilution 200 µL were applied on cells in duplicates and incubated for 1h at 37°C in 5% CO_2. Supernatant was removed, cells washed with 1x PBS and overlaid with 500 µL of 2x MEM supplemented with 2% penicillin/streptomycin and 4.4 g/L $NaHCO_3$ / 2.4% Avicel (60:40) Cells were incubated for 4 days at 37°C in 5% CO_2. Overlay was removed, cells fixed in 6% Pfa for 20 minutes and stained with crystal violet solution for 10 minutes. Depending on virus concentration, plaques were counted for all countable dilutions and the titre was calculated according to the following equation

$$[Pfu/ml] = \frac{\Sigma \; plaques \; x \; dilution \; factor}{\Sigma \; applied \; volume}$$

2.2.2.10. Virus kinetic

VeroFM, EidNi, EidLu, Ma104 and A549 cells were seeded in 6-wells (8×10^5 cells), infected with a MOI of 0.001 and incubated for 1h at 37°C. Supernatant was removed, cells washed with 1x PBS and incubated in supplemented DMEM. Supernatants and cells infected with Kumasi rhabdovirus were collected at 0, 8, 24, 48, 72, and 96h post infection for RNA extraction and virus titration. RNA extraction and real-time RT-PCR were done as described above.

2.2.3. 454 sequencing of KRV

Full genome sequence of Kumasi rhabdovirus was done by *de novo* sequencing using 454 Life Sciences technology. A T163 flask Vero FM cells was infected with a MOI of 0.01 with Kumasi rhabdovirus and incubated at 37°C and 5% CO_2 for two days. Supernatant was harvested and cells lysed by one freeze-thaw cycle. Cells and supernatant were clarified at 300 x g for 5 minutes. Supernatant was applied to a

succhrose gradient ranging from 10-60% and ultracentrifuged with a SW40 Ti rotor at 28,000 x g for 21h at 4°C. Fractions of 0.5 mL were collected, analysed by real-time RT-PCR (2.2.6.2.) and fractions with highest viral RNA concentration were pooled. Pooled fractions were clarified at a 36% succhrose cushion at 28,000 x g for 4h at 4°C, the pellet dissolved in 500 μL PBS overnight and viral RNA extracted according to (2.2.5.4.).

RNA was extracted using viral RNA kit without carrier RNA (Qiagen). RNA was reverse transcribed and double-strand cDNA was sythesized using a cDNA Synthesis System Kit (Roche). 500 ng of double stranded (ds) cDNA was fragmented according to the Roche GS Junior Rapid Library Preparation Method Manual. Fragment ends were repaired, 454 sequencing adaptors were ligated and emulsion PCR was performed according to the standard 454 sequencing protocols (Roche). Next generation sequencing of the library was performed with a Genome Sequencer Junior (Roche).

Reads were *de novo* assembled using the Newbler software (Roche) and resulting contigs were aligned against the non redundant NCBI database with the blastn, blastx and tblastx algorithms [100].

The novel rhabdovirus sequence was confirmed by generating overlapping RT-PCR amplicons using primers that were designed based on a 10,982 bp long contig that showed similarities to other rhabdovirus sequences. Sequencing of RT-PCR products was performed using the Sanger method. Genome ends were generated by use of a GeneRacer Kit (LifeTech).

2.2.4. Serological methods

2.2.4.1. Enzyme-linked-immunosorbent assay (ELISA)

Bat and human samples were pre-screened with an in-house enzyme-linked immunosorbent assay (ELISA) for antibodies against KRV. 96-well plates (NuncMaxiSorp) were coated with 0.4 μg virus antigen in 0.1 M $NaHCO_3$-buffer (pH

9.6) and blocked with 5% milk powder in PBS 0.1% Tween20. Sera were diluted 1:100 in 1% milk powder in 1x PBS 0.1% Tween20 and incubated for two hours. Detection was done with goat-anti-bat immunoglobulin (Ig) horseradish peroxidase (HRP) labeled (Dianova, Hamburg, Germany) (1:2,000), goat-anti-human (Ig) HRP labeled (Bethyl Laboratories Inc.) (1:4,000), respectively. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added, reaction stopped with 2 M sulfuric acid and absorption measured at 450 nm and 630 nm on either Synergy 2 Multi-Mode Microplate reader (BioTek) or Spectramax 190 (Molecular Device). Data were analysed with Gen5 or SOFT max Pro 3.0 software. Cut-off was calculated by tripling the optical density values of the negative control.

2.2.4.2. Indirect immunofluorescence assay (IFA)

Bat, livestock and human sera were screened with a KRV specific immunofluorescence assay. VeroE6 cells were infected with an MOI = 0.1 in supplemented DMEM and incubated for two days. Cells were mixed 1:1 with non-infected cells (1.2×10^6 cells/mL) spottet on 12-well immunoslides (Dunn Labortechnik GmbH), air-dried and fixed in ice-cold methanol:acetone (1:1). Sera were diluted 1:40 and detection was done with either goat-anti-bat antibody Ig (Bethyl Laboratories) 1:1,000 followed by donkey-anti-goat cyanine 2 (Cy2) labeled Ig (Dianova) 1:100 for bat samples or goat-anti-human Cy2 labeld Ig (Dianova) 1:400 for human samples or Goat-anti-swine Alexa Fluor488 Ig (Dianova), goat-anti-bovine Alexa Fluor488 Ig (Dianova), Donkey-anti-sheep Alexa Fluor488 Ig (Dianova) 1:200, respectively for livestock samples. Slides were analysed and pictures taken with a Motic fluorescence microscope (Zeiss).

2.2.4.3. Plaque-reduction-neutralization assay (PRNT)

Sera were heat inactivated at 56°C for 30 minutes. All sera were assayed two in parallel in two-fold dilutions ranging from 1:10 to 1:320 for the final dilutions. Dilutions were mixed with approximately 23 PFU per well and incubated for 1h at 37°C, 5% CO_2. 1×10^5 VeroFM cells in supplemented DMEM were seeded in 48-well plates, incubated overnight and washed once with 1x PBS before addition of 100 µL

serum-virus dilution. After 1h incubation at 37°C, 5% CO_2 supernatant was removed, cells washed with 1 x PBS and overlaid with 2.4% Avicel/2x MEM supplemented with 4,4 g/L $NaHCO_3$, 20% FCS and 2% penicillin-streptomycin (40:60) and incubated for four days at 37°C, 5% CO_2. Thereafter, cells were washed with 1x PBS, fixed with 6% paraformaldehyde for 20 minutes and the cell layer stained with crystal violet. Plaques were counted and the 50% neutralization titre calculated. Neutralizing titres of 1:10 were interpreted as borderline.

2.2.4.4. Determination of protein concentration

Protein concentration was determined according to Bradford Assay [102]. A serial dilution of albumin ranging from 2 mg/ml to 0.0312 mg/mL was used as standard. In brief, 12.5 μL of sample was mixed with 375 μL CoomasiePlus™ Protein Assay Reagent and incubated at room temperature for 10 minutes. Absorption was measured at 595 nm in a photometer and concentration determined by extrapolation in the standard curve.

2.2.5. Molecular biological methods

2.2.5.1. Isolation of viral RNA from tissue and mosquitoes

Viral RNA was isolated with QIAamp RNeasy kit (Qiagen) according to manufacturer's instruction. In brief, approximately 50 mg tissue, 10 mosquitoes respectively were mixed with 600 μL lysis buffer containing 6 μL β-mercaptoethanol. Samples were homogenized at 30 1/s for 3 minutes in a tissue lyser (Qiagen). Samples were incubated for 2 minutes on ice, 600 μL 70% ethanol was added, mixed, applied to columns and centrifuged at 8,000 x g for 30 seconds. Membrane was washed with 700 μL RW1 and 500 μL RPE with a spin at 8,000 x g for 30 seconds. The final wash was done with 500 μL RPE followed by centrifugation at 20,000 x g for 2 minutes. Column was dried at 20,000 x g for 5 minutes and RNA eluted with 100 μL 80°C RNase-free water at 8,000 x g for 1 minute after incubation for 4 minutes. Samples not directly processed were stored at -80°C.

2.2.5.2. Isolation of viral RNA from serum

RNA from serum samples was isolated with QIAamp MinElute Virus Spin Kit (Qiagen) according to manufacturer's instruction. In brief, 140 μL serum was mixed with 25 μL QIAGEN Protease and 200 μL AL buffer and incubated at 56°C for 10 minutes. 5.6 μg carrier-RNA and 250 μL ethanol were added, incubated at room temperature and applied to the column. The column was centrifuged at 6,000 x g for 1 minute, the membrane washed with 500 μL AW2 buffer, centrifuged at 6,000 x g for 1 minute, washed with 500 μL ethanol and centrifuged at 20,000 x g for 10 minutes. For elution, 100 μL 80°C hot AVE was added, incubated at 4°C for 4 minutes and centrifuged at 20,000 x g for 1 minute. RNA was stored at -80°C until use.

2.2.5.3. Isolation of viral RNA from urine

Viral RNA was isolated with QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's instruction. In brief, 140 μL sample was mixed with 560 μL AVL containing 5.6 μg carrier RNA and incubated for 10 minutes at room temperature. 560 μL ethanol was added to sample, applied to column and centrifuged at 6,000 x g for 1 minute. The membrane was washed with 500 μL AW1, centrifuged at 6,000 x g for 1 minute and 500 μL AW2 centrifuged at 20,000 x g for 3 minutes. Column was dried at full speed for 5 minutes and samples were eluted with 60 μL AVE heated to 80°C after incubation for 1 minute at 6,000 x g for 1 minute. Samples not used immediately were stored at -80°C for later use.

2.2.5.4. Isolation of viral RNA from cell culture supernatant

Viral RNA was isolated with NucleoSpin® RNA virus according to manufacturer's instructions. In brief, 75 μL samples were mixed with 300 μL RAV1 and inactivated at 70°C for 5 minutes. The sample was mixed with 300 μL ethanol, applied to column and centrifuged at 8,000 x g for 1 minute. The membrane was washed with 500 μL RAW and 600 μL RAV3 with a spin of 8,000 x g for 1 minute in between. Final wash was done with 200 μL RAV3, the membrane dried twice at 11,000 x g for 5 minutes

and RNA eluted with 60 μL 80°C RNase-free water after an incubation of 1 minute at 11,000 x g for 1 minute.

2.2.5.5. Isolation of total RNA from cells

Total RNA was isolated from cells with NucleoSpin® RNA II according to manufactures instructions. In brief, cells were lysed in 350 μL RA1 containing 3.5 μL β-mercaptoethanol. Lysates was applied to filter columns and spined at 11,000 x g for 1 minute. Binding conditions were adjusted by adding 350 μL 70% ethanol to the sample, mixture applied to column and spined at 11,000 x g for 30 seconds. Membranes were desalted with 350 μL MDP, centrifuged at 11,000 x g for 1 minute and DNA digested with 95 μL DNAse reaction mixture at room temperature for 15 minutes. Membranes were then washed with 200 μL RA2 and 600 μL RA3 and centrifuged at 11,000 x g for 30 seconds in between. The final wash was done with 250 μL RA3 at 11,000 x g for 2 minutes and membranes were dried at 11,000 x g for 4 minutes. RNA was eluted in 60μL RNase free water at 80°C by centrifuging at 11,000 x g for 1 minute. Samples were stored at -20°C until needed

2.2.5.6. Agarose gel electrophoresis

Electrophoretic analysis of DNAs on agarose gels was done to verify PCR amplification. Gel electrophoresis was done with 2% RotiAgarose GTQ in 1x TBE buffer containing 1.5 μL of 1% ethidium bromide per 50 mL agarose. 10 μL of PCR product was mixed with 6x loading dye, applied on gel and separated at 80-120 V. The size of DNA fragments was determined by using a 100 bp DNA ladder. After separation of DNA, the gel was analysed under UV light.

2.2.5.7. Purification of PCR products

PCR products were purified for further analysis with SeqLab purification kit. PCR product was mixed with 500 μL binding buffer, applied to column and centrifuged at

10,000 x g for 2 minutes. Elution buffer (20-50 µL) was applied, incubated for 5 minutes and purified DNA eluted at 5,000 x g for 1 minute.

2.2.5.8. Photometric determination of nucleic acid concentration

Nucleic acid concentrations were determined using PeqLabs NanoDrop 2000c. As a blank, solvent of nucleic acid was used. For photometric determination, 1 µL of nucleic acid was measured three times and the mean value used as concentration.

2.2.5.9. Sequencing of DNA

Purified PCR product (2.2.5.7.) were sent to SeqLab Sequence Laboratories Göttingen for sequencing. Sequencing reaction contained 2 µL purified DNA, 1.43 µmol/L sequencing primer and 4 µL RNase free water. Sequence data was viewed with BioEdit 7.0.5.3 and sequence alignments were generated with DNASTER Lasergene 7.

2.2.5.10. Generation of *in vitro* transcript

For the quantification of Kumasi rhabdovirus RNA an *in vitro* transcript was generated. The amplicon from a 893 bp fragment of the N-gene was purified according to 2.2.5.7. and cloned with TOPO® TA Cloning® kit (Invitrogen). The Ligation reaction contained 2 µL of amplicon, 0.5 µL salt-solution and 0.5 µL pCA TOPO TA cloning vector and was incubated for 30 minutes at room temperature, followed by 2 minutes on ice. For transformation, 50 µL TOP 10 competent cells were added to the ligation reaction and incubated on ice for 25 minutes. Heat-shock was done for 30 seconds at 42°C followed by 1 minute on ice and bacteria were thereafter incubated in 250 µL SOC medium at 37°C for 1h. Transformed bacteria were plated on LB-Agar plates containing kanamycin and incubated at 37°C over night. Orientation of insert was tested with a colony PCR. Reaction mix contained 2.5 µL 10x Platinum Taq buffer, 200 nmol/L dNTP each, 2 mmol/L $MgCl_2$, 19.4 µL RNA free water, 200 nmol/L M13mod forward and BtRhabdoM17-rt reverse primer and half of bacteria

colony. Cycling conditions were 6 minutes at 95°C with 40 cycles of 15 seconds at 05°C, 15 seconds at 58°C and 40 seconds at 72°C with a final elongation of 1 minute at 72°C. PCR products were analysed by gel electrophoresis (2.2.5.6). Clones with the correct orientation of insert were expanded in 1 mL LB-medium containing kanamycin at 37°C over night. Plasmid DNA was extracted with QIAprep Spin Miniprep-Kit (Qiagen) according to manufacturer's instructions. In brief, overnight culture was centrifuged at 17,000 x g for 2 minutes and the pellet resuspended in 250 μL P1-buffer. Suspension was carefully mixed with 250 μL P2-buffer followed by 350 μL N3-buffer and cleared at 17,000 x g for 10 minutes. Supernatant was applied on QIAprep-column, spinned at 17,000 x g for 1 minute, washed with 500 μL PB-buffer and 750 μL PE-buffer with a spin of 17,000 x g for 1 minute followed and dried with an extra spin. Plasmid DNA was eluted in 50 μL EB-buffer after an incubation of 1 minute at room temperature at 17,000 x g for 1 minute. Isolated DNA was serially diluted from 10^{-1} to 10^{-12} in RNase-free water containing 10 ng/μL carrier RNA and amplified with M13mod forward and reverse primers and 5 μL template as described above. The last detectable dilution was sequenced (2.2.5.9.) for confirmation and *in vitro* transcribed with MegaScript T7 ® kit (Invitrogen). The *in vitro* transcription mix contained dNTP 7.5 mmol/L each, 2 μL 10x reaction buffer, 2 μL enzyme mix, 4 μL RNase free water and 4 μL PCR product. The transcription mixture was incubated at 37°C for 4h followed by a DNA digest with 1μL TurboDnase at 27°C for 30 minutes. The transcript was purified with the RNeasy kit (Qiagen) according to manufactors "clean-up" protocol and eluted in 50 μL RNase free water. RNA concentration was measured according to 2.2.5.8. and the copy number/μL calculated according to the following equation

$$[Molecules/\mu l] = \frac{RNA\ concentration\ g/L}{transcript\ length\ bp \times 340\ g/mol} \times (6 \times 10^{23}\ mol^{-1})$$

The transcriped was aliquoted and stored at -20°C until use.

2.2.6. Reverse transcription polymerase chain reaction

Polymerase chain reaction (PCR) is generally used for exponential amplification of DNA fragments using a DNA polymerase and specific forward and reverse primers. In One-step reverse transcription (RT) PCR the same principle applies however, RNA is first transcribed into cDNA using a reverse transcriptase followed by amplification of the cDNA. The reaction is done in one tube, thereby minimizing the risk of contamination and simplifying the reaction setup.

2.2.6.1. Genera specific hemi-nested RT- PCR for *Paramyxoviridae*

Paramyxoviridae were detected with a genera specific hemi-nested RT PCR according to [101]. The assay covers the genera *Respirovirus/Morbillivirus/Henipavirus, Avulavirus/Rubulavirus* and the genus *Pneumorvirus* in three independent reactions. Full virus RNA extracts of Measles-, Mumps- and Human metapneumo virus were used as controls for the individual reactions. Reactions were done in 25 μL using the Invitrogen Superscript™ III OneStep RT-PCR kit with 12.5 μL 2x reaction buffer, 920 nmol/L first round forward and reverse primer, 2.4 mmol/L $MgSO_4$, 200 μmol/L dNTP each, 1 μL enzyme mix, 1 μg BSA, 10 units (U) RNAseOut (Invitrogen) and 5 μL RNA template. First round amplification involved 1 minute at 60°C, 30 minutes at 48°C, 2 minutes at 94°C followed by 45 cycles of 15 seconds at 94°C, 30 seconds at 50°C and 30 seconds at 72°C followed by a final elongation time of 7 minutes at 72°C. The second round of PCR was done in 50 μL using 2 μL of first round PCR product, with 5 μL 10x Platinum Taq buffer (Invitrogen), 200 μmol/L dNTP each, 2.0 mmol/L $MgCl_2$, 1 μmol/L second round forward and reverse primer, and 1 U Platinum Taq polymerase. Second round amplification was done for 3 minutes at 94°C with 45 cycles of 15 seconds at 94°C, 30 seconds at 50°C and 30 seconds at 72°C with a final elongation of 7 minutes at 72°C. PCR products were analysed by gel electrophoresis (2.2.5.6.).

2.2.6.2. Kumasi rhabdovirus Real-time RT PCR

Kumasi rhabdovirus RNA was detected and quantified by real-time RT PCR. The assay sensitivity was determined to be on the order of 90 copies per reaction by using a quantified *in vitro* transcript (2.2.5.10.). Reactions were done in 25 μL using the Invitrogen Superscript™ III OneStep RT-PCR kit with 12.5 μL 2x reaction buffer, 1.6 mmol/L $MgSO_4$, 200 μmol/L deoxynucleoside triphosphates (dNTP), 800 nmol/L forward and reverse primer, 280 nmol/L Kumasi rhabdovirus specific probe, 1μg bovine serum albumin (BSA), 0.8 μL RNA-free water, 1 μL enzyme mix and 5 μL RNA template. Reverse transcription was done at 55°C for 20 minutes, followed by initial denaturation at 95°C for 3 minutes and 45 cycles of 95°C for 15 seconds and 58°C for 40 seconds, a final cooling round at 37°C for 2 minutes was included.

2.2.6.3. *Henipavirus* real time RT-PCR

A subset of spleen positive for the genus Henipa virus and the organs of the respective animals were quantified by real time RT-PCR. Depending on the primer combination minor changes were done in the protocol. The general reaction was done in 25 μL using the Invitrogen Superscript™ III OneStep RT-PCR kit with 12.5 μL 2x reaction buffer, 1.6 mmol/L $MgSO_4$, 200 μmol/L deoxynucleoside triphosphates (dNTP), 800 nmol/L forward and reverse primer, 280 nmol/L probe, 0.5 μg bovine serum albumin (BSA), 0.8 μL RNA-free water, 1 μL enzyme mix and 5 μL RNA template. Reverse transcription was done at 55°C for 20 minutes, followed by initial denaturation at 95°C for 3 minutes and 45 cycles of 95°C for 15 seconds and 58°C for 40 seconds, a final cooling round at 37°C for 2 minutes was added. Viral concentration was calculated by extrapolation, assuming the minimum of detection is 10 viral RNA copies at a cycle threshold (CT) of 40.

2.2.7. Phylogentic analyis

2.2.7.1. Phylogenetic analysis KRV

Reference sequences of *Rhabdovirdiae* were downloaded from GenBank (accession numbers are provided in Fig. 2) and protein coding sequences were extracted using Geneious 5.1 software [103]. Translated amino acid sequences were aligned using

MAFFT V7 [104]. Tree topologies were calculated with MEGA 5.2 [105] using the Maximum Likelihood algorithm with the complete deletion option and a WAG+G+I+F predicted to be the most suitable using the Model selection tool in MEGA 5.2. Bayesian phylogeny of dimarhabdoviruses was done with MrBayes V.3.1 [106] also using the WAG amino acid substitution model with 1,000 parallel Maximum Likelihood bootstrap replicates. Trees were visualized in FigTree from the BEAST V1.7.4 package [107], Rabies virus was chosen as outgroup.

2.2.7.2. Phylogenetic analysis *Paramyxoviridae*

Reference sequences of *Paramyxoviridae* were downloaded from GenBank (accession numbers are provided in Fig. 4). Translated amino acid sequences were aligned using MAFFT V7 [104]. The alignment contained a 439 nucleotide (henipaviruses) and 169 nucleotide (rubulaviruses) part of the L-gene. Bayesian phylogenetic trees of henipa- and rubulaviruses were done with MrBayes V.3.1 [106] using the WAG amino acid substitution model. Statistical support of grouping from Bayesian posterior probabilities contained 1,000 parallel Maximum Likelihood bootstrap replicates. Measles virus was chosen as outgroup for henipaviruses and Nipah virus as outgroup for rubulaviruses. For rubulaviruses, a monophyly prior was set on all members of the genus *Rubulavirus* to stabilize the phylogenetic reconstruction. Trees were visualized in FigTree from the BEAST V1.7.4 package [107]. The maximum internal amino acid distance among henipa- and rubulavirus clades was calculated in MEGA5.2 using the pairwise deletion option.

2.2.8. Statistical analysis

Cross tables were calculated using EpiInfo V7 (www.cdc.gov/epiinfo). Student's T-test and ANOVA were calculated using IBM SPSS Statistics 22. Comparisons were considered significant when corrected 2-tailed p values were below 0.05.

3. Results

3.1. Sampling

In this study, *E. helvum* was sampled over three consecutive years (2009-2012) in Kumasi, Ghana for the purpose of detecting new and potential human pathogens, that could emerge from a bat reservoir, and observe them on a longitudinal base. *E. helvum* is a migratory bat and resides in Kumasi mainly from October to April. Therefore, the sampling was conducted during these months, with an exception of the years 2009 and 2011, where additional samples were available in June also. Occasionally, the colony left town and the intended number of 12 animals per week was not sampled. For December 2009 and January 2012, no samples were available for the same reason. In total, 630 *E. helvum* were captured and sacrificed during the sampling time. For each individual bat, the spleen, liver, kidney, brain, gut and intestine were sampled and aliquots of each sample were shock-frozen and formalin embedded. Additionally, urine samples from the same colony were collected. Of 155 urine samples, 17 were pooled samples and contained 10 drops of urine each. Human serum samples were obtained from 45 individuals working at and/or living in the zoological gardens of Kumasi or handling bats on a regular basis. Furthermore, 103 samples from patients of the Komfo Anokye Teaching Hospital in Kumasi were obtained from a co-operation partner and included in the study. Livestock serum was sampled from 106 cattle, 105 sheep, 124 goat and 107 swine. Samples were collected from farms and abattoirs in and around Kumasi.

3.2. Detection of *Paramyxoviridae* in *E. helvum*

The spleen of 630 *E. helvum* from Kumasi, Ghana, sampled during the years 2009 to 2012 were tested by three hemi-nested RT-PCR [101] assays designed to allow detection of all *Paramyxoviridae* genera. The spleen was used for screening because several virus-positive animals had shown the highest virus concentrations in the spleen. Additionally, 155 urine samples from the same colony were tested. Henipaviruses were detected in 109 (17.3%) animals while rubulaviruses were detected

Table 1: **Detection rate of *Paramyxoviridae* in *E. helvum*.** The samples were collected over three consecutive years in Kumasi, Ghana. The table below shows the total number of samples tested, the number and percentage of spleen tested positive for the genera *Henipa-* and *Rubulavirus*. Months in which no positive sample was detected are marked with minus.

Month	Year	Tested spleens	Henipavirus	%	Rubulavirus	%
March	2009	23	7	30.4	6	26.1
June	2009	20	3	15	3	15
November	2009	60	17	28.3	4	6.7
January	2010	13	-	-	-	-
February	2010	18	1	5.6	-	-
March	2010	37	4	10.8	9	24.3
April	2010	6	-	-	1	16.7
October	2010	20	5	25	2	10
November	2010	40	5	12.5	-	-
December	2010	5	-	0	-	-
January	2011	22	1	4.6	-	-
February	2011	17	3	17.6	2	11.7
March	2011	48	8	16.7	9	18.7
April	2011	32	10	31.3	13	40.6
Mai	2011	22	4	18.2	2	9.1
June	2011	10	1	10	1	10
October	2011	43	4	9.3	3	6.9
November	2011	48	8	16.7	6	12.5
December	2011	49	5	10.2	4	8.2
February	2012	46	16	34.8	8	17.4
March	2012	36	3	8.3	-	-
April	2012	15	4	26.7	-	-
Total		**630**	**109**	**17.3**	**73**	**11.6**

in 73 (11.6%) animals. In none of the samples pneumoviruses were detected. This data set included samples (2009 to 2010), already published, in which 31 henipaviruses and 20 rubulaviruses were detected [16]. Additional 71 henipaviruses and 84 rubulaviruses were found in samples from 2010 to 2012.

3.3. Phylogeny of *Paramyxoviridae* in *E. helvum* and other African fruit bats

Phylogenetic trees were constructed which contained all *Paramyxoviridae* sequences from the *E. helvum* colony in Kumasi and all published henipa- and rubulaviruses from African fruit bats [16, 108, 109]. The sample set covered the countries Ghana, Gabon, Republic of Congo, Central African Republic and Democratic Republic of the Congo.

In a bayesian analysis, henipaviruses clustered in eleven well-supported clades (Fig. 4A). In agreement with the phylogeny, the clades could be separated by a 30% amino acid distance threshold (Fig. 4C), seven of which contained viruses from *E. helvum* of Kumasi. Two other clades contained viruses only from other fruit bats (*Myonyteris, Epomophorus, Hypsignathus, Rousettus, Megaloglossus*). Another two clades contained the Australian Hendra- and Nipahvirus and Cedarvirus, respectively. Viruses present in *E .helvum* from Kumasi were also found in *E. helvum* from the five different sampling countries.

Rubulaviruses from African fruit bats clustered in ten clades in a bayesian analysis (Fig. 4B). In accordance with phylogeny, these clades could be separated by a 31% amino acid detection threshold. Eight of these clades contained viruses of *E. helvum* from Kumasi. Achimota virus 2 (AchV2) was recently isolated from *E. helvum* in Accra, Ghana and formed a single clade. This virus was not detected in *E. helvum* from Kumasi although it is assumed that the colony in Accra and Kumasi belongs to the same population of bats. Tuhoko virus 2 (TukV2) was detected in a Chinese *Rousettus* bat and also clustered individually in the present phylogenetic tree. Rubulaviruses detected in *E. helvum* from Kumasi were also detected in all five sampling countries. Seven of theses rubulaviruses were not published previously.

The sampling of *E. helvum* from Kumasi was done during three consecutive years (2009 to 2012). All but one henipavirus clade detected in *E. helvum* bats were present in the Kumasi colony in all three sampling years. For rubulaviruses, all clades which contained viruses from *E. helvum* were represented in Kumasi apart from AchV2.

The viral concentrations of henipaviruses were determined for 22 positive animals with real-time RT-PCR. Virus concentrations in the spleen ranged from 10^5 to 10^8 viral RNA copy numbers per gram tissue (Fig. 5). The RNA concentrations were comparable over the years 2009 to 2011 to a mean of $10^{6.51}$ viral RNA copy numbers per gram tissue. Viral RNA was also detected in the liver, kidney, lung, intestine and serum, but the highest concentrations were in the spleen (Fig. 5 and Tab. 2).
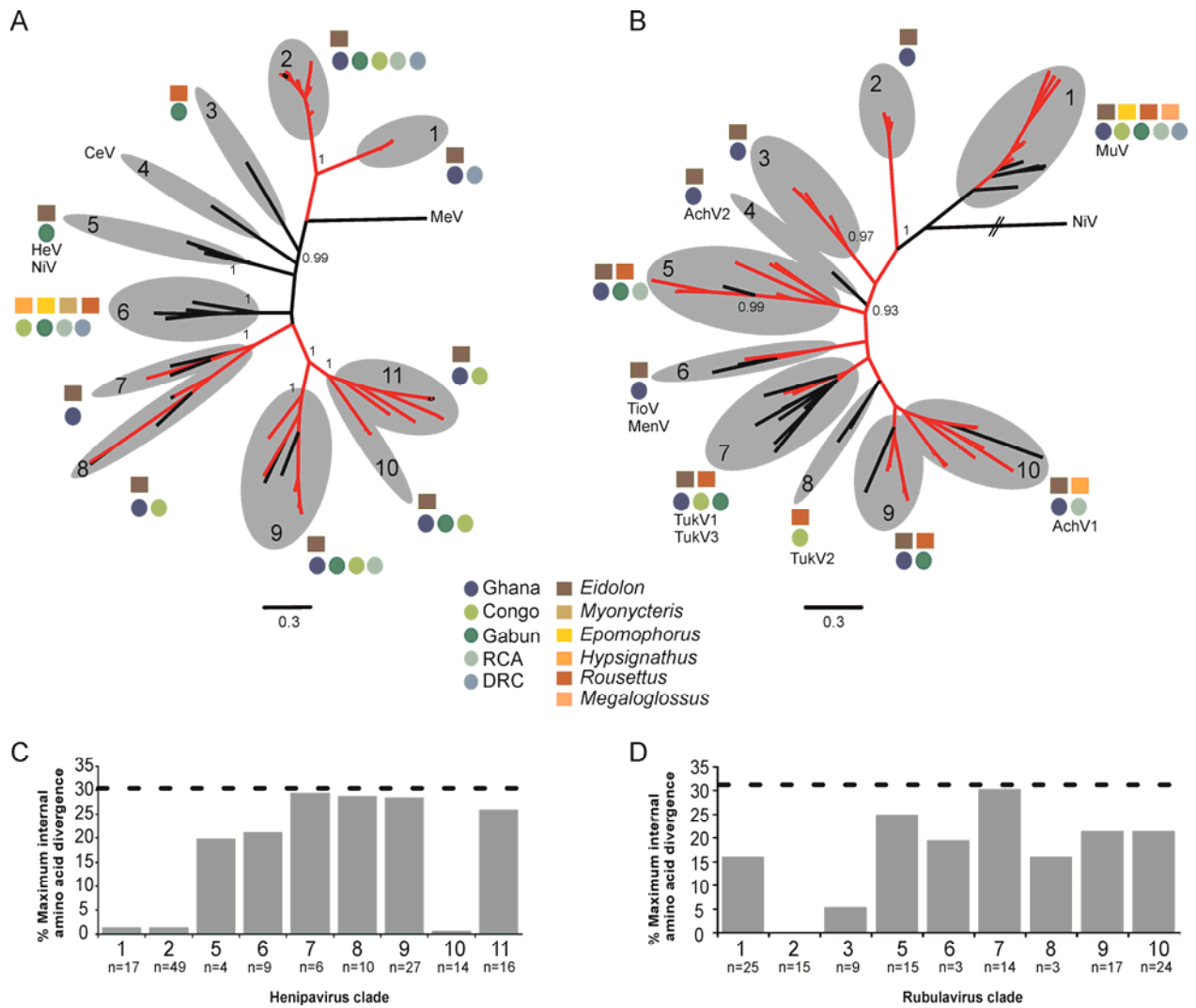
Figure 4: **Bayesian phylogeny and maximum internal amino acid distance of the L-gene of bat paramyxoviruses.** (A) Henipaviruses detected in African fruit bats (B) Rubulaviruses detected in African fruit bats. The statistical support for posterior are not shown for rubulavirus clades because these clades were identified in Neighbour-Joining phylogeny. Circles represent sampling area (RCA = Central African Republic, DRC = Democratic Republic of the Congo, Congo = Republic of the Congo), squares indicate fruit bat species. Viruses detected in *E.helvum* from Kumasi are highlighted with red branches. Numbers represent individual clades. Branch lengths are drawn to scale. Statistical support of grouping from Bayesian posterior probabilities and 1,000 parallel Maximum Likelihood bootstrap replicates is indicated at deep node points. Scale bars correspond to genetic distance. Internal amino acid divergence of the L-fragment is shown for henipavirus clades (C) and rubulavirus clades (D). Maximum amino acid sequence distance was calculated with MEGA5.2 using the pairwise deletion option. Clades which contained only one sequence were excluded for graphical reasons.

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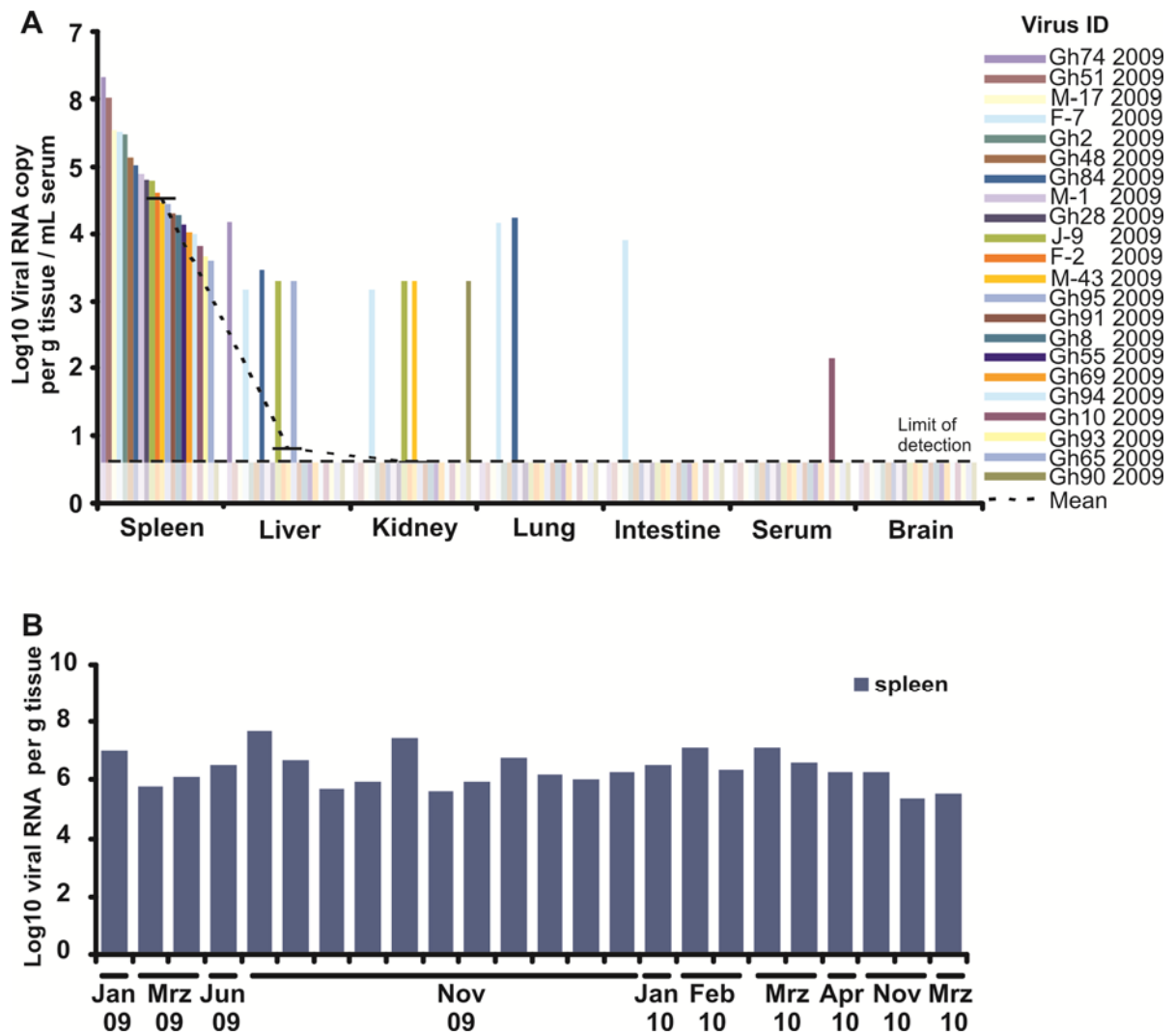
Figure 5: **Henipavirus RNA concentrations in solid organs of *E. helvum* bats.** (A) Virus concentrations given in log10 RNA copies per mL of serum, or per gram of tissue scaled on the y-axis, were plotted for each bat organ tested. All bats were sampled between 2009-2010 from one large colony located in Kumasi, Ghana. To facilitate evaluation of virus concentrations, organs were structured according to means, which were connected with a solid line for graphical reasons only. Bars below the limit of detection of the real time RT-PCR assay, used for quantification, represent negative test results. Colours represent individual bats given on the right of the figure. (B) Viral concentration of paramyxoviruses in the spleen of individual bats, shown in A, but are represented over the sampling period 2009 to 2010.

Table 2: **Henipavirus viral RNA concentrations in bat solid organs and serum.** Bats were sampled between 2009 and 2011. Virus concentrations are given in log10 RNA copies per gram tissue or millilitre serum. Samples in which viral concentration was below the detection limit are marked with minus.

	Log10 viral RNA copies per g tissue / mL serum						
Animal ID	Spleen	Liver	Kidney	Lung	Intestine	Serum	Brain
Gh74	6.32	4.18	-	-	-	-	-
Gh51	6.02	-	-	-	-	-	-
M-17	5.55	-	-	-	-	-	-
F-7	5.52	3.18	3.18	4.16	3.91	-	-
Gh2	5.48	-	-	-	-	-	-
Gh48	5.14	-	-	-	-	-	-
Gh84	5.02	3.48	-	4.24	-	-	-
M-1	4.89	-	-	-	-	-	-
Gh28	4.80	-	-	-	-	-	-
J-9	4.79	3.31	3.31	-	-	-	-
F-2	4.61	-	-	-	-	-	-
M-43	4.47	-	3.31	-	-	-	-
Gh95	4.45	3.31	-	-	-	-	-
Gh91	4.31	-	-	-	-	-	-
Gh8	4.28	-	-	n.a.	n.a.	-	n.a.
Gh55	4.14	-	-	-	-	-	-
Gh69	4.02	-	-	-	-	-	-
Gh94	4.00	-	-	-	-	-	-
Gh10	3.82	-	-	-	n.a.	2.15	-
Gh93	3.67	-	-	-	-	-	-
GH65	3.60	-	-	-	-	-	-
GH90	-	-	3.31	-	-	-	-

n.a. = no sample available

3.4. Virus isolation

Virus isolation from *E. helvum* spleen and urine samples was approached in two ways: as undirected virus isolation, without knowing the virological background of the sample, and as directed virus isolation with samples positive for the genus *Henipavirus*. No virus isolate was obtained from directed virus isolation from neither spleen nor urine samples. From ten spleens used in undirected virus isolation, a virus was isolated
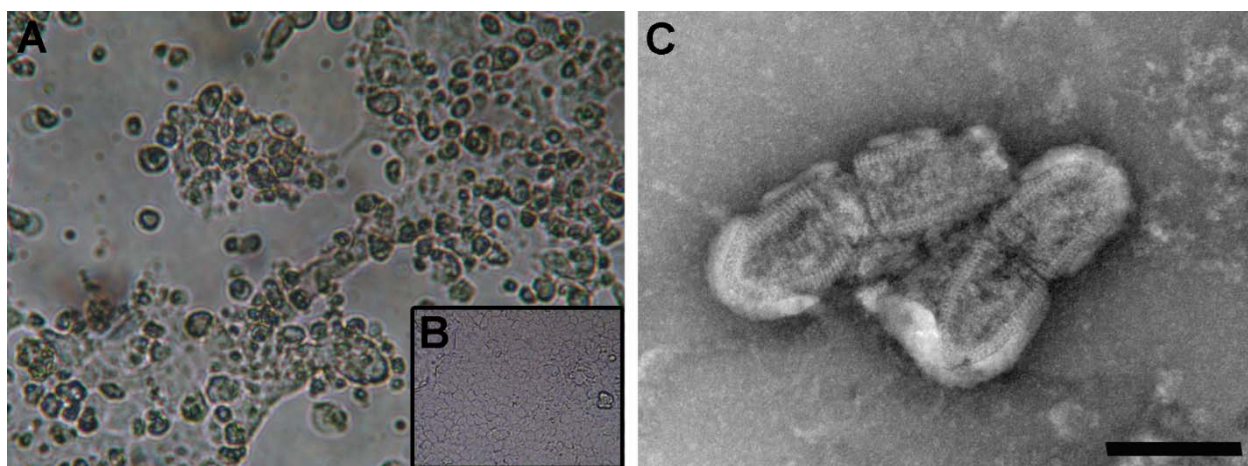
Figure 6: **Cytopathic effect and electron microscopic (EM) picture of KRV.** VeroE6 cells were infected with KRV with an MOI of 0.001. (A) Cytopathic effect of KRV four days post-infection. VeroE6 cells were infected with an MOI of 0.001 after four days cells detached and rounded. (B) Mock control: VeroE6 cells monolayer after four days. (C) EM picture of KRV. The supernatant of KRV infected VeroE6 cells was ultracentrifuged on a saccharose cushion and the pellet was used for analysis. The bar in the EM picture represents 100 nm. The picture was courtesy of A. Kurth.

from one spleen collected in March 2010 (M35). Four days post infection (dpi), a CPE characterized by rounding and detaching of cells, was seen on EidNi/EidLu cells (Fig. 6A). The supernatant of infected cells revealed particles with the characteristic bullet shape of rhabdoviruses by electron microscopy (Fig. 6C). The virus was named Kumasi rhabdovirus (KRV) after the town of its isolation.

3.5. Virus characterisation

The growth of KRV on primate, human and bat cell lines was characterized. Virus concentration was determined by real-time RT-PCR and plaque assay and expressed as log10 viral RNA copies/mL and log10 pfu/mL. African green monkey kidney interferon incompetent (VeroFM) and competent (Ma104) cell lines, human lung carcinoma cell line (A549) and *E. helvum* kidney (EidNi) and lung (EidLu) cell lines were used. KRV could be propagated in all tested cell lines with similar growth kinetics (Fig. 7). KRV reached the growth plateau between 48 and 72 h with the
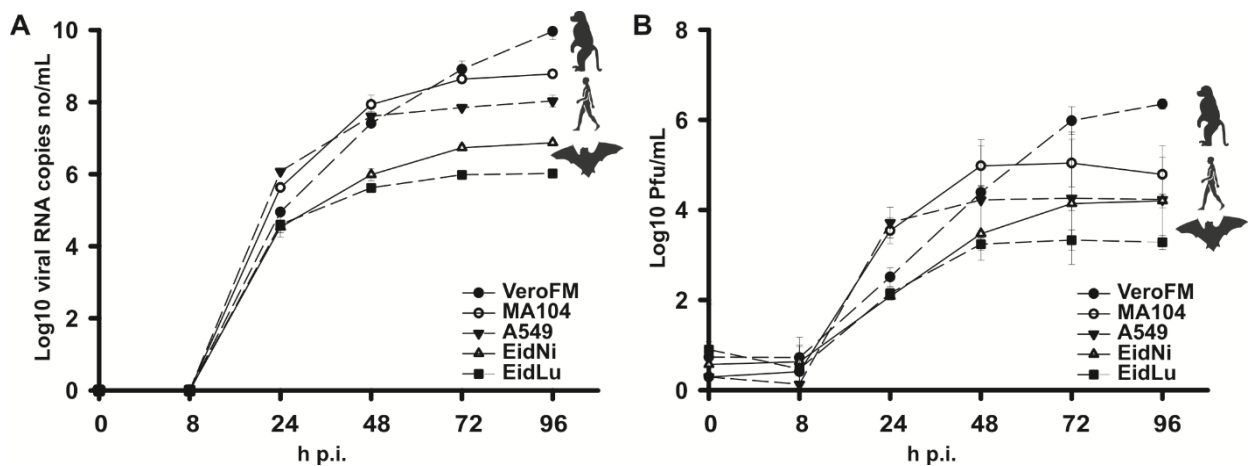
Figure 7: **Growth kinetic of KRV on mammalian cell lines**. KRV was inoculated with a MOI of 0.001 on African green monkey kidney interferon incompetent (VeroFM) and competent (Ma104) cell lines, human lung carcinoma cell line (A549) and *E. helvum* kidney (EidNi) and lung (EidLu) cell line. Virus concentrations are given in log 10 RNA copies/mL (A) and log 10 pfu/mL (B). KRV enters and replicates in all five cell lines. Highest titres were reached in primate cell lines, followed by the human cell line and bat cell lines.

exception of VeroFM cells in which the plateau was reached after 96 h. Virus concentration varied between species. On primate cell lines, the highest titers of KRV were obtained on interferon incompetent cells. In comparison, growth on human cell lines was reduced to 1.8 - 42.4 fold. The lowest titres were seen in bat cell lines with better replication on kidney cell cultures, compared to lung cell cultures.

3.6. Detection of KRV

Screening for the presence of KRV in *E. helvum* organs was done with specific real-time RT-PCR. The spleen was used because several virus-positive animals had shown the highest virus concentrations in the spleen. *E. helvum* spleen from three consecutive seasons (2009-2011) were tested. KRV was detected in 25 (5.1%) of 487 spleens. Virus concentration ranged from 10^4 to 10^8 viral RNA copy numbers per gram tissue. For all positive animals, viral RNA concentrations in the lung, brain, liver, kidney, gut, serum and intestine were determined with real-time RT-PCR (Fig. 8 and Tab. 3). The predominance of virus concentrations in the spleen was confirmed. In five animals, viral RNA was also detected in other organs.
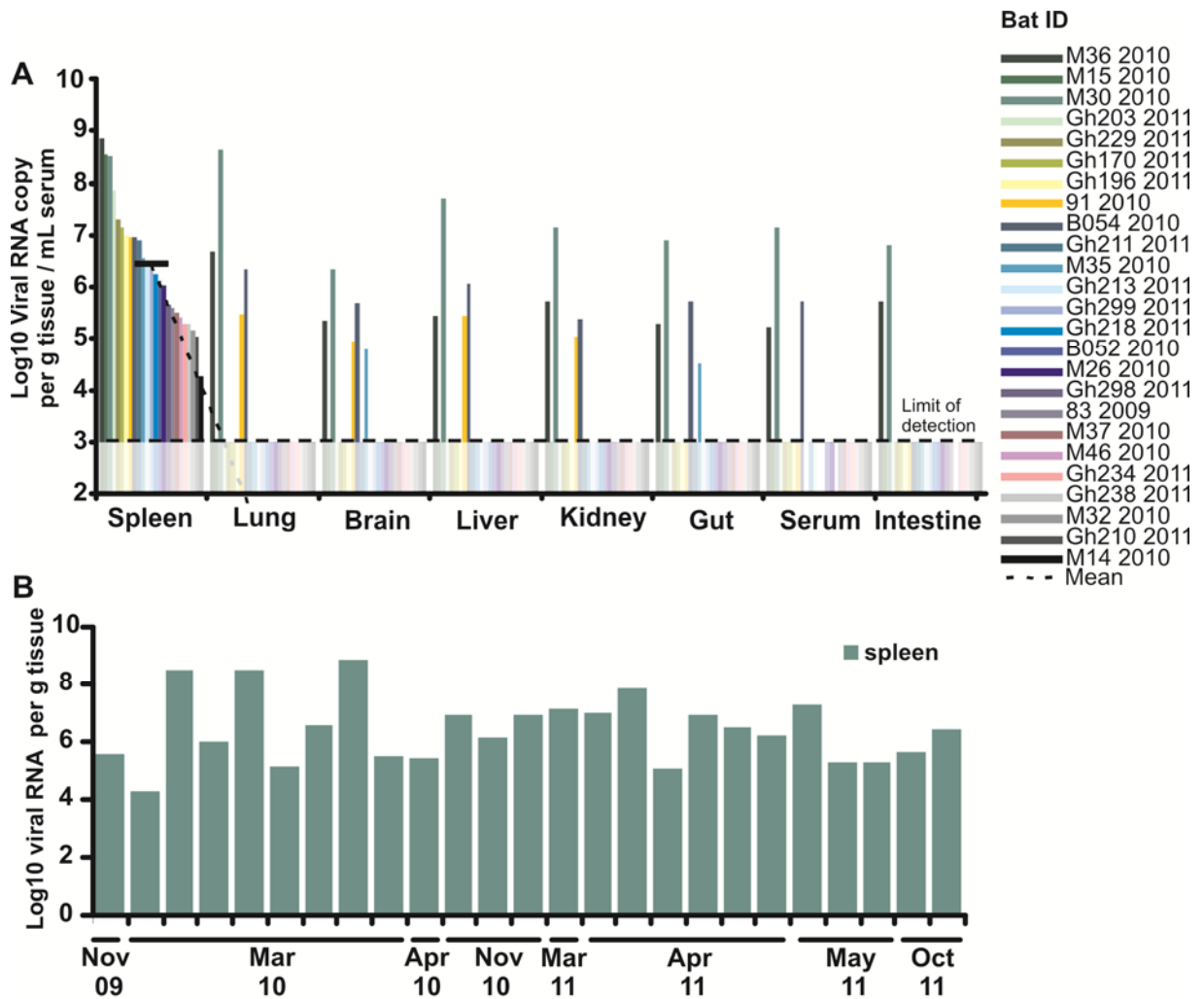
Figure 8: **KRV RNA concentrations in solid organs of *E. helvum* bats.** (A) Virus concentrations given in log10 RNA copies per mL of serum, or per gram of tissue scaled on the y-axis, were plotted for each bat organ tested. All bats were sampled between 2009-2011 from one large colony located in Kumasi, Ghana. To facilitate the evaluation of virus concentrations, organs were structured according to means, which were connected with a solid line for graphical reasons only. Bars below the limit of detection of the real time RT-PCR assay used for quantification represent negative test results. Colours represent individual bats given on the right of the figure. (B) Viral concentration of KRV in the spleen of individual bats, shown in A, but are represented over the sampling period 2009 to 2011.

Table 3: **KRV RNA concentrations in solid organs of *E. helvum* bats.** Bats were sampled between 2009 and 2011. Virus concentrations are given in log10 RNA copies per gram tissue or millilitre serum. Samples in which viral concentration was below the detection limit are marked with minus.

Animal ID	Log10 viral RNA copies per g tissue / mL serum							
	Spleen	Lung	Brain	Liver	Kidney	Gut	Serum	Intestine
M36	8.85	6.70	5.36	5.44	5.72	5.28	5.22	5.71
M15	8.53	-	-	-	-	-	-	-
M30	8.52	8.62	6.33	7.72	7.15	6.91	7.14	6.80
Gh203	7.86	-	-	-	-	-	-	-
Gh229	7.31	-	-	-	-	-	-	-
GH170	7.15	-	-	-	-	-	-	-
Gh196	6.99	-	-	-	-	-	-	-
91	6.96	5.46	4.93	5.44	5.04	-	-	-
B054	6.95	6.35	5.68	6.05	5.39	5.72	5.73	-
Gh211	6.89	-	-	-	-	-	n.a.	-
M35	6.55	-	4.81	-	-	4.51	-	-
Gh213	6.47	-	-	-	-	-	-	-
Gh299	6.44	-	-	-	-	-	n.a.	-
Gh218	6.25	-	-	-	-	-	n.a.	-
B052	6.12	-	-	-	-	-	-	-
M26	6.03	-	-	-	-	-	-	-
Gh298	5.66	-	-	-	-	-	n.a.	-
83	5.60	-	-	-	-	-	-	-
M37	5.52	-	-	-	-	-	-	n.a.
M46	5.42	-	-	-	-	-	-	-
Gh234	5.29	-	-	-	-	-	-	-
Gh238	5.29	-	-	-	-	-	-	-
M32	5.16	-	-	-	-	-	-	-
Gh210	5.04	-	-	-	-	-	-	-
M14	4.26	-	-	-	-	-	-	-

n.a. = no sample available

Viral concentrations in the spleen were comparable over the sampling months and years to a mean concentration of $10^{6.44}$ viral RNA copies per gram tissue (Fig. 8 and Tab.3). The detection rate varied in the sampling years but no significant difference in seasons (p = 0.72) or year were observed (p = 0.73) (Tab. 5). KRV was detected in the months of March, April, May, October and November, but not in January, February, June and December. There were no samples available for the months of July, August and September, due to migration of the colony. The detection rate of KRV was significantly higher in juveniles compared to adults (χ^2 = 4.06, p = 0.04).

Due to the known tropism of some rhabdoviruses for the central nervous system, 630 brains were separately screened for KRV by RT-PCR, irrespective of the testing outcome of spleen samples. No additional positive brain samples were found.

As it is known that many rhabdoviruses are transmitted by and maintained in arthropod vectors, 1240 female mosquitoes (*Aedes* ssp., *Culex* ssp., *Eretmapodites* ssp., *Lutzia* ssp., *Mansonia* ssp., *Toxorhynchites* ssp.) from close proximity of the *E. helvum* colony sampled in 2011, were tested in real-time RT-PCR. None of the tested mosquitoes was positive for KRV viral RNA.

3.7. Phylogenetic classification of KRV

The phylogenetic classification of *Rhabdoviridae* is classically done using the *nucleoprotein* (N) gene. In figure 9 a phylogenetic tree of the N-gene, including all genera of *Rhabdoviridae,* is shown. Because highest sequence conservation and better support were obtained when the *L-protein* (L) gene was used, phylogenetic analysis of KRV was done using the L-gene.

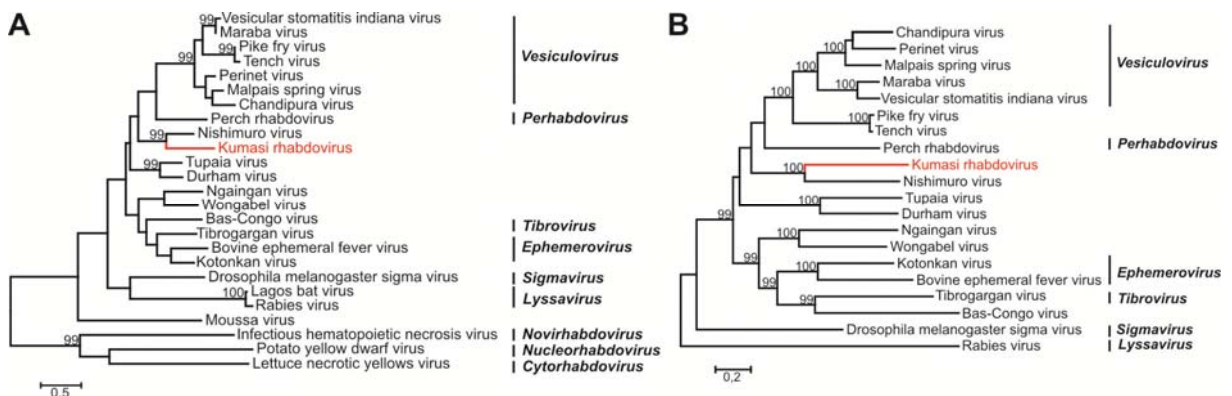Figure 9: **Phylogentic relationship of the N and L protein of KRV** (red). Analyses were performed using the Maximum likelihood algorithm. Tree settings are described in methods. Branch lengths are drawn to scale. (A) Phylogenetic tree of the N protein including representatives of all *Rhabdoviridae* genera. (B) Phylogenetic relation of the L protein of members of the dimarhabdovirus supergroup.

Figure 10A shows a Maximum likelihood tree of the L-gene including all *Rhabdoviridae* genera. KRV was classified in the supergroup of dimarhabdovirses. It clustered in an independent, deeply-rooted branch monophyletic to Nishimuro virus. This relationship was confirmed in a tree of the N-gene (Fig. 9A).

The dimarhabdovirus supergroup comprises viruses with only short sequence information available for the L-gene. Therefore, a Bayesian analysis of dimarhabdoviruses, using the N-gene, was done. KRV clustered in a well supported clade, monophyletic with Mount Elgon bat virus (MEBV), Oita rhabdovirus (OIRV) and Kern Canyon virus (KCV) (Fig. 10B). This relation was supported with high amino acid identities in the L-gene (71.3 – 88.9) and the N-gene (41.2 – 65) (Tab. 4). Nishimuro virus formed a sister group to this clade.

3.8. Genome characterization of KRV

The complete 11,075 nt KRV genome exhibited typical rhabdovirus organization consisting of 3'- and 5' terminal non-coding regions (approximately 70 nt and 28 nt, respectively) and five genes, each bound by putative transcriptional regulatory sequences. As shown for other rhabdoviruses the transcription inititiation sequence (AACGAGA) and transcription termination sequence (CAUG$[A]_7$) are highly conserved with exception of gene 2 whose termination sequence is CUGG$[A]_6$. Gene 1 (N) encodes a 427-aa protein (47.95 kDa), gene 3 (M) encodes a 207-aa protein (23.81 kDa), gene 4 (G) encodes a 551-aa protein (62.02 kDa), and gene 5 (L) encodes a 2122-aa protein (241.55 kDa). No continuous orf was found in gene 2. However, three smaller orfs were predicted coding for three hypothetical proteins with 72 aa (orf 2), 22 aa (orf 3), and 57 aa (orf 4).
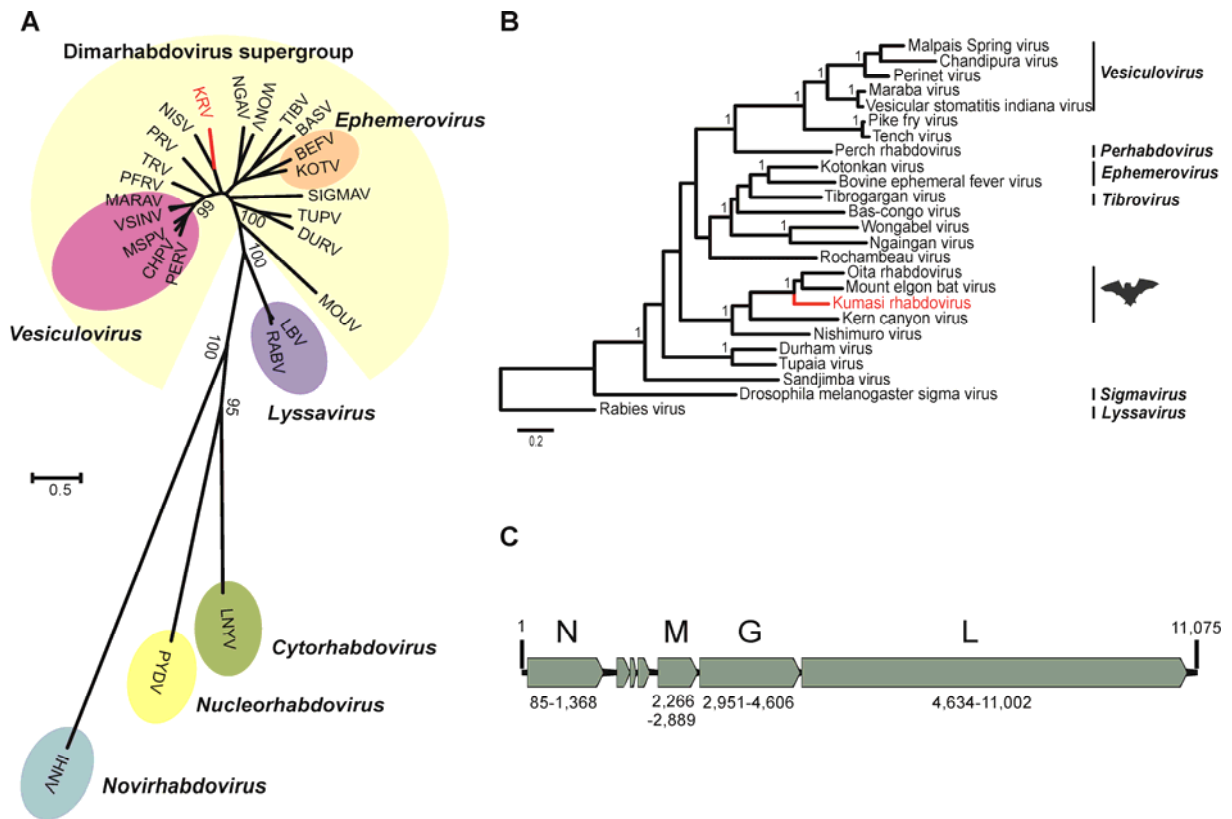
Figure 10: **Phylogentic classification and genome organisation of KRV**. (A) Maximum likelihood analysis of the L gene including representatives of all *Rhabdoviridae* genera and KRV (red). (B) Bayesian tree of the N-genes of the dimarhabdovirus supergroup. The WAG amino acid substitution model was used. The statistical support of grouping from Bayesian posterior probabilities and 1,000 parallel Maximum Likelihood bootstrap replicates are indicated at deep node points. Scale bars correspond to genetic distance. (C) Genome organization of KRV. Grey arrows represent suggested open reading frames (orf) for putative nucleocapsid (N), matrix (M), glyco (G) and large (L) protein along the KRV genome. Also shown are three hypothetical orfs that were predicted in the genome region between N and M. Numbers below orf symbols represent genome positions of the respective orf. BASV (JX297815) Bas-Congo virus, BEFV (NC002526) Bovine ephemeral fever virus, CHPV (NC020805) Chandipura virus, SIGMAV (NC013135) Drosophila melanogaster sigma virus, DURV (FJ952155) Durham virus, IHNV (NC001652) Infectious hematopoietic necrosis virus, KCV (DQ457101) Kern Canyon virus, KOTV (NC017714) Kotonkan virus, KRV (KJ179955) Kumasi rhabdovirus, LBV (NC020807) Lagos bat virus, LNYV (NC007642) Lettuce necrotic yellows virus, MSPV (KC412247) Malpais spring virus, MARAV (HQ660076) Maraba virus, MEBV (DQ457103) Mount Elgon bat virus, MOUV (FJ985749) Moussa virus, NISV (AB609604) Nishimuro virus, NGAV (NC013955) Ngaingan virus, OIRV (AB116386) Oita rhabdovirus, PRV (NC020803) Perch rhabdovirus, PERV (HM566195) Perinet virus, PFRV (FJ872827) Pike fry virus, PYDV (NC016136) Potato yellow dwarf virus, RABV (NC001542) Rabies virus, (DQ457104) Rochambeau virus, (DQ457102) Sandjimba virus, TRV (KC113517L) Tench virus, TIBV (NC020804) Tibrogargan virus, TUPV (NC007020) Tupaia virus, VSINV (NC001560) Vesicular stomatitis Indiana virus, WONV (NC011639) Wongabel virus.

Table 4: **Amino acid identity of individual protein sequences of the dimarhabdovirus supergroup**. Identities are expressed in percent and were calculated as pairwise deletion using MEGA 5.1. Sequence information was not available for every gene of each virus.

Pairwise amino acid identities

L gene	KRV	MEBV	ORV	KCV	NISV	VSINV	CHPV	BEFV	KOTV	PFRV	PRV	TUPV	WONV
KRV													
MEBV*	86.1												
ORV	88.9	83.3											
KCV**	71.3	83.3	83.3										
NISV	47.6	86.1	80.6	74.3									
VSINV	43.7	72.2	66.7	67.6	44.9								
CHPV	43.7	66.7	61.1	66.2	43.7	59.7							
BEFV	38.1	63.9	61.1	61.0	40.5	41.8	41.9						
KOTV	40.9	61.1	61.1	63.2	41.9	44.7	43.2	50.6					
PFRV	43.4	77.8	66.7	69.9	42.9	52.8	52.5	42.1	44.0				
PRV	41.7	66.7	69.4	71.3	44.0	45.5	45.7	41.0	41.7	46.9			
TUPV	39.7	55.6	52.8	63.2	41.2	41.8	41.5	38.5	39.0	40.5	40.3		
WONV	38.9	61.1	58.3	61.0	40.8	41.4	42.1	42.3	43.8	42.8	41.8	41.3	

*identity over 281 amino acid residues **identity over 316 amino acid residues

N gene	KRV	MEBV	ORV	KCV	NISV	VSINV	CHPV	BEFV	KOTV	PFRV	PRV	TUPV	WONV
KRV													
MEBV*	65.0												
ORV*	59.2	63.1											
KCV**	41.2	42.3	42.5										
NISV	39.7	44.0	36.7	42.2									
VSINV	27.9	31.4	30.3	30.4	30.7								
CHPV	27.6	27.9	28.1	30.7	30.8	51.0							
BEFV	29.4	32.3	29.3	32.1	31.7	30.6	30.0						
KOTV	30.1	32.6	29.3	35.9	34.1	32.0	31.3	51.8					
PFRV	32.1	33.7	30.5	31.4	29.9	52.8	45.6	30.7	29.2				
PRV	28.8	33.8	28.5	34.0	30.7	32.3	31.1	30.3	32.0	32.8			
TUPV	30.8	36.6	32.5	34.6	37.2	30.8	29.8	31.4	35.4	32.2	28.7		
WONV	28.7	30.1	30.0	31.4	32.3	30.3	29.8	35.8	38.1	28.8	27.3	32.6	

*identity over 36 amino acid residues **identity over 136 amino acid residues

G gene	KRV	NISV	VSINV	CHPV	BEFV	KOTV	PFRV	PRV	TUPV	WONV
KRV										
NISV	25.7									
VSINV	21.3	22.0								
CHPV	21.9	24.1	40.6							
BEFV	20.5	19.0	18.8	20.2						
KOTV	19.5	17.9	22.2	19.8	31.8					
PFRV	20.2	19.8	33.6	31.7	18.8	19.5				
PRV	20.2	21.5	23.3	23.7	17.9	19.1	23.4			
TUPV	20.2	21.4	19.3	20.6	15.6	16.9	19.6	18.5		
WONV	23.2	21.3	20.5	22.8	21.9	22.4	20.8	22.0	18.6	

M gene	KRV	NISV	VSINV	CHPV	BEFV	KOTV	PFRV	PRV	TUPV	WONV
KRV										
NISV	32.0									
VSINV	10.2	17.7								
CHPV	11.9	18.2	29.4							
BEFV	23.7	24.1	12.9	10.6						
KOTV	22.1	24.0	11.9	13.4	41.0					
PFRV	12.8	15.9	32.0	23.4	14.8	15.2				
PRV	17.2	19.5	16.3	13.0	18.9	21.1	15.5			
TUPV	15.8	15.9	10.1	12.5	16.1	16.5	9.8	16.3		
WONV	18.3	19.3	11.2	13.1	21.2	25.6	14.0	20.1	10.9	

3.9. Seroprevalence of KRV

3.9.10. *E. helvum*

The overall prevalence of KRV in the bat population was investigated by antibody detection assays. Of 349 *E. helvum* sera, pre-screened with an in-house ELISA 182 (52.1%) had OD values above threshold (data not shown). The samples were confirmed with IFA on slides containing virus infected cells. Additional 127 serum samples were tested only with IFA. Representative pictures of IFA of positive and negative sera are shown in Fig. 11. Antibodies against KRV were detected in 55 (11.5%) *E. helvum* serum samples. Positive samples were confirmed with PRNT. Neutralizing antibodies were detected in 33 sera (6.4%) in the range of 1:360 to 1:10. There was no significant difference in gender ($\chi^2 = 1.3$, p = 0.26) or age ($\chi^2 = 0.03$, p = 0.87) distribution. In 2010, 18 (13.5%) animals had neutralizing antibodies compared to 2009, 4 (3.9%) and 2011, 11 (4.8%) (Tab. 5).

3.9.10. Livestock

Cross species transmission of KRV was tested for cattle, sheep, goat and swine from Kumasi and the surrounding with IFA. None of 106 cattle, 105 sheep, and 124 goat sera reacted with KRV. Out of 107 swine sera, 27 (28.9%) reacted with KRV in IFA. Five of these serum samples (5.4%) were confirmed with PRNT. However, neutralizing activity in all cases was at the limit of titration of the assay, yielding 50% plaque reduction at dilutions equal to 1:10. Representative pictures of IFA for positive and negative swine sera are shown in Fig. 11.

3.9.11. Human

Transmission of KRV to humans was tested for 163 samples with IFA. Samples originated from inhabitants of Kumasi with 45 serum samples from people living or
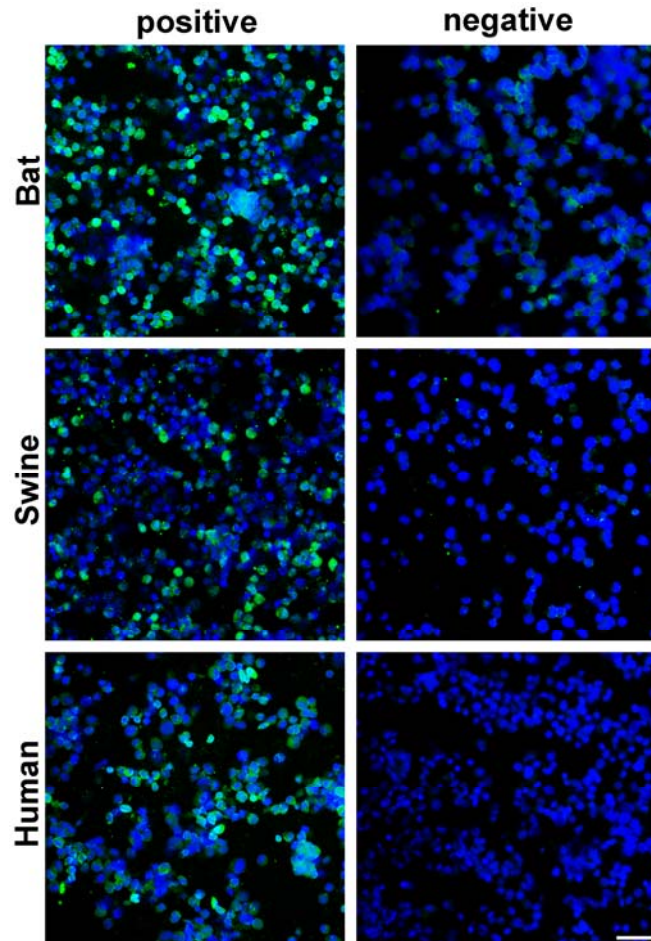
Figure 11: **Serological analysis of bat, livestock and human sera for antibodies to KRV** with specific indirect immunofluorescence assay. Vero FM cells infected with KRV were mixed with uninfected cells (1:1) and spotted on glass slides. Sera were diluted 1:40. Examples of sero positive and negative *E. helvum*, swine and human sera are shown here. Detection was done with goat anti-bat immunoglobulin (Ig), followed by donkey anti-goat Ig labeled with cyanine 2 (Cy2), goat-anti-swine Ig labelled with Cy2, goat-anti-human Ig labelled with Cy2, respectively. All positive samples had neutralizing antibodies in a 50% plaque-reduction assay. The bar represents 50 μm.

working in close proximity to the *E. helvum* colony, and 118 samples from patients of the Komfo Anokye Teaching Hospital (KATH) which were provided by a co-operation partner. Six sera showed reactivity in IFA. Five of these samples originated from workers of the Zoological gardens of Kumasi, but none of them had neutralizing antibodies against KRV. One patient sample had neutralizing antibodies with a titre of 1:20. Positive and negative IFA pictures are presented in Fig. 11.

Table 5: **Detection rate and seroprevalence of *E. helvum* for KRV**. Samples were collected over three consecutive years in Kumasi, Ghana. The total numbers of samples tested for KRV are shown. The number and percentage of spleen tested positive with specific real time RT-PCR and the number and percentage of sera with neutralizing antibodies. Months in which no sample was positive are marked with minus.

Month	Year	Total number spleen/sera	Viral RNA	%	Neutralizing antibodies	%
January	2009	6/0	-	-	n.a.	
March	2009	16/20	-	-	3	15
June	2009	19/18	-	-	1	5.6
November	2009	55/56	1	1.8	2	3.6
January	2010	12/11	-	-	2	18.2
February	2010	17/16	-	-	1	6.3
March	2010	33/37	8	24.2	4	10.8
April	2010	4/6	1	25	1	16.7
October	2010	40/38	-	-	7	18.4
November	2010	18/17	3	16.7	1	11.8
December	2010	5/11	-	-	1	9.1
January	2011	18/12	-	-	-	-
February	2011	16/17	-	-	2	11.8
March	2011	44/41	1	2.3	3	4.9
April	2011	32/32	6	18.8	2	6.3
May	2011	22/17	3	13.6	2	11.8
June	2011	10/4	-	-	1	25
October	2011	37/42	2	5.4	-	-
November	2011	48/46	-	-	-	-
December	2011	37/35	-	-	-	-
Total		**487/467**	**25**	**5.1**	**33**	**6.4**

n.a.= sample not available

4. Discussion

4.1. Virus diversity and potential viral origin

The potential of bats to act as sources or reservoirs of emerging viruses is a complex problem amenable to analysis on various levels of investigation. Important aspects include ecological factors such as virus diversity and abundance, epidemiological factors such as the opportunities for interspecies contact, as well as virological parameters such as replication level and population immunity. In this work, two aspects were highlighted and investigated, including virus diversity in a fruit bat colony and the transmission of bat-derived viruses to humans and livestock. Fruit bats harbour a variety of virus families, wherein this study focused on members of the families *Paramyxoviridae* and *Rhabdoviridae* [41-43].

Within the family *Paramyxoviridae* most interesting findings were obtained for the genera *Henipa-* and *Rubulavirus*. Members of these genera cause relevant infectious diseases in animals and humans (e.g. Rinderpest virus, Peste-des-petits-ruminants virus, Measles virus and Mumps virus). Our group has recently presented fundamental data linking the evolutionary provenance of all of those viruses to bats [16]. Additionally, some of the known fruit bat specific henipa- and rubulaviruses are known to cause recurring outbreaks in animals and humans making them fulfil the definition of zoonotic agents, such as Henda-, Nipah and Menangle virus [64, 76, 95].

Theoretically, the complete diversity of viruses in our samples could have been captured by deep sequencing. However, this method is still cost-intensive and currently does not feature the sensitivity necessary for testing complex samples such as organ biopsies. Also less complex samples such as serum or urine will not yield any results if the viral load is low. For this reason a hemi-nested RT-PCR was chosen as the main tool to test for novel paramyxoviruses in this study. The disadvantage of RT-PCR methods is an amplification bias caused by the choice of primers. Expectably, more known viruses will be detected in comparison to novel viruses. To compensate at least partly for primer-dependent bias, a combination of three different hemi-nested

RT-PCR assays were applied, which were each designed so as to provide optimized specificity of one of the three genera *Henipavirus*, *Rubulavirus*, and *Morbillivirus*. These assays were known for their sensitivity, covered a broad range and were already successfully used for the detection of novel viruses [37, 108]. Furthermore, virus isolation was attempted for the detection of novel paramyxoviruses but remained unsuccessful. The isolation of unknown viruses from wild-life is challenging as no standard procedures can be applied. Successful virus isolation depends on a variety of factors such as the choice of cell culture systems, the concentration of virus input, conditions for maintenance of cell cultures, as well as the use of additives such as serum or growth hormones. Despite the availability of *E. helvum* cell cultures, knowledge of the other factors was lacking, posing challenges in the interpretation of negative results.

In previous studies, paramyxoviruses have been detected in faeces, urine and the spleen of bats [16, 37, 108]. Urine and faeces do not involve invasive sampling and enable the collection of large amounts of samples. But urine and droppings are collected from a plastic foil which is placed under a roosting tree, and it is therefore not possible to assign samples to individual animals. Multiple-time sampling of individuals as well as some limited extent of cross-contamination between samples can therefore not be excluded. Working with organ samples can circumvent this problem but it involves killing of animals, limiting the number of samples that can be taken. I have followed a sampling design in which only few animals per visit had to be sacrificed while care was taken not to disturb the colony, with regular monitoring of signs of disturbance such as displacement of individual sub-groups to another known nearby roost in the same city. All organs from a subsample of sacrificed animals were initially tested quantitatively for henipaviruses, confirming the expectation that highest virus concentrations should be detectable in the spleen. In the remaining investigation, testing was then focused on spleen samples. Along with the targeted invasive sampling, non-invasive faecal and urine samples were taken from the colony during each visit. I believe this approach provides an overview of the diversity of viruses by collecting many non-invasive samples, and enables clear insight into detection rates by providing access to organs from individual animals.

Another way to improve the quality of my sample was to include diverse sources of sequence data. In my phylogenetic analyses, sequences from this study were supplemented by sequences from six different African fruit bat species sampled in five different African countries during own previous studies, as well as a number of published datasets [16, 37, 108, 109]. These collated data contain the largest overview on henipa- and rubulavirus genetic diversity possible at this time point.

An immense new diversity of henipa- and rubulaviruses in Ghanaian fruit bats was detected in this study. Overall, the diversity of viruses remained constant over the three years of my observation. Remarkably, nearly the complete diversity of henipa- and rubulaviruses was represented in a single *E. helvum* colony from Kumasi, indicating that the viruses may be shared among fruit bats in Africa during annual migrations. There are currently no data available for the diversity of paramyxoviruses in other *E. helvum* colonies, making it impossible to say whether this colony is unique among others. However, the data suggest a remarkably high virus diversity in *E. helvum* in general. The reasons are unclear and it would be interesting to investigate the virus diversity from isolated colonies and compare the results with the diversity detected in this study [38].

E. helvum is a migratory species which crosses large geographic distances (> 2000 km) during its annual migration [22]. Spatiotemporal overlap and social mixing of colonies might promote virus exchange. Accordingly, species with none or a small migratory range would exhibit lower virus diversity. In my data the virus diversity within the genus *Rubulavirus* was evenly distributed across bat species, indicating that the sample was large enough to display virus diversity up to a level of saturation (it was shown in a recent study that only a certain number of samples is necessary to detect the proximate virus diversity of a given species [110]). Interestingly, in the henipavirus phylogeny viral diversity was far larger in *E. helvum* than in any other species, supporting the idea of migratory behaviour to potentially foster the acquisition of a large range of viruses.

On the contrary, population size and density exert evolutionary pressure on parasites [111] and thus could as well influence the evolution of virus diversity. *E. helvum* forms huge colonies with up to 1 million animals while most other African fruit bats roost in smaller groups or individually. *Rousettus* is the only other species known to form social groups of considerable size, but these will typically not exceed 50,000 individuals [112]. Social groups can be dense, as *Rousettus* roosts in caves. However, if population density is the main factor driving virus diversity, the rate of henipavirus and rubulavirus diversity should have been similar in *Rousettus* bats, which was not the case.

The differences in host diversity for henipa- and rubulaviruses might arise from varying host spectra. Some genera as for example *Avulavirus* have a narrow host range while others such as *Morbillivirus* have a broad host range [44]. Therefore it is possible that the detection of rubulaviruses in a variety of fruit bat species accounts for a generally broader host range of this virus genus compared to henipaviruses. This is supported by the detection of rubulaviruses not only in fruit bats but also in insectivorous bats [16, 113]. Henipaviruses were detected in fruit bats only, indicating a restriction to this host family. It should be mentioned, however, that there is some unconfirmed and preliminary evidence for potential existence of henipaviruses in two insectivorous bat species [78, 114]. A sampling bias can be excluded as it would have affected both genera to the same extend.

Due to the rather clear restriction of henipaviruses with pteropodid bats, virus-host co-evolution seems at least conceivable. Co-evolution was shown for some DNA viruses which deemed to evolve at slower pace, such as the papillomaviruses seemingly co-evolve with their feline hosts. Here phylogenetic trees of virus and host species are topological congruence [115].

An indication for a virus lineage to have evolved (i.e., to stem) from a certain host species is if viruses from the host species of interest are simultaneously detected at high and deep notes of the virus phylogeny [16]. In my dataset such a double-representation could be seen for henipaviruses in *E. helvum* and, to a much lesser extent, spanning less

patristic viral distance, in *Rousettus*. Those few henipavirus clades from other fruit bats which cluster together and are not co-detected in *E. helvum* might have evolved from *E. helvum* and might still be detectable in *Eidolon* if the sample size was increased. If we accept considerations made in [110], only approximately 85% of henipavirus diversity from *E. helvum* should have been detected in the sample size analyzed in this study. However, it needs to be mentioned that the projections as well as the fundamental analysis made in that publication are highly controversial, and not widely accepted. Likewise it is possible that the virus lineage which was the origin of this cluster is already extinct in *E. helvum*. It should also be considered that the sample volume for other fruit bats in this study was large but not equal to that for *E. helvum* - a bias which could not be adjusted for in the limited scope of this virological investigation.

One particular clade of henipaviruses which includes the Australian and Asian Hendra- and Nipah virus species is of special interest. Also in this clade, viruses from *E. helvum* were represented, which raises questions regarding a potential African origin of henipaviruses. The highest diversity of henipaviruses was detected in Africa compared to Asia and Australia. An overlap of habitats between the African and Asian bats is known [116], and it is therefore possible that the Asian and Australian bats acquired their viruses from Africa originally. More importantly it was shown that the African henipaviruses share a conserved GDNQ motif in their RNA-dependent RNA polymerase which is thought to be part of the catalytic side for polymerase. This motif is shared among most *Paramyxoviruses* and even most *Mononegavirales*, therefore represents a bona-fide ancestral motif. On the contrary, the Asian henipaviruses have an atypical GDNE motif which is also present in some of the Eidolon-associated henipaviruses (while other Eidolon-associated henipaviruses have the ancestral motif) [16]. This taken together with the virus diversity detected in Africa is indicative of an African origin of henipaviruses.

In summary, the data presented here give evidence for a possible co-evolution of henipaviruses and *E. helvum*. However, they do not explain the origin of the henipaviruses from other fruit bats. A scenario is imaginable where henipaviruses have co-evolved with *E. helvum* but occasionally transferred to other fruit bat species. The

original lineages leading to this events might be invisible to us because they are already extinct in *E. helvum* and/or the new host.

This study was focused on *E. helvum* and available sequences from other fruit bat species are currently not sufficient for extensive phylogenetic calculations. In the future, when more sequence information was generated, the calculations done in this study should be repeated and complemented with parsimonious calculations to obtain a more thoroughly picture of the relation of fruit bats and henipaviruses.

4.2. Transmission of viruses from *E. helvum*

A variety of viruses were detected in *E. helvum* but the possibility of spill-over to other species is still unclear. Evidence for cross-species transmission is difficult to obtain by direct virus detection. Serological investigation of potential target species such as livestock or human is required. However, these investigations classically requires a virus isolate as surrogate techniques such as viral pseudotypes can only partly display the processes of viral neutralization and need to be validated on full virus assays. Isolation of paramyxoviruses was attempted but remained unsuccessful, and the lack of isolates so far has prevented classification of viruses into serotypes.

In the framework of this study I have used another virus as a surrogate for potential virus transmission from *Eidolon*, namely a *rhabdovirus* that was isolated from the *E. helvum* colony in Kumasi. The virus was classified in the supergroup of dimarhabdoviruses and named Kumasi rhabdovirus (KRV). To obtain indications for possible transmission pathways, the virus concentration in all organs was evaluated and high detection rates as well as concentrations were found in the spleen. Henipaviruses are also present at high concentrations in the spleen and their transmission is probably *via* urine and/or saliva [80, 87]. Urine samples were not available at the time of testing but a subset of salivary glands were tested negative for KRV. Many rhabdoviruses replicate in the nervous system but KRV was only detected at low rates and low concentrations in brains of *Eidolon* individuals. High detection rates of KRV in the spleen and low detection rates in the brain suggests the

involvement of the lymphatic system rather than the nervous system in virus infection. In contrast, rabies virus was found at high titres in the brain of bats but only to limited extent in the spleen [117]. Persistence and long-term replication of viruses in lymphoid organs was observed [118] therefore replication of KRV in the spleen is possible. The spleen has three main functions, including the proliferation and storage of lymphocytes as well as the removal of aged erythrocytes from the blood. In this context viral particles may be carried from the blood stream or other compartments into the spleen and simply accumulate there. The role of the spleen as a potential place of KRV replication therefore needs further confirmation. Histological methods might identify virus budding in the spleen to confirm the place of replication. For all *E. helvum* organs, formalin fixed samples are available and offer opportunities for further investigation.

Studies of virus transmission require proof of presence of the virus in donor species. In our study, the time of sampling encompassed three years and KRV was detected in 5.1% of the tested animals. The detection rates of KRV coincided with the seasons in Ghana, pointing to actual ongoing transmission between bats. During wet seasons (March to July and September to November), viral detection rate increased while KRV was not detected during the dry Harmattan season (December to February). Most hosts develop a permanent protective immunity and virus maintenance depends on herd immunity and the recruitment rate of young animals. Accumulation of susceptible hosts at nesting colonies can increase virus amplification and the risk of transmission and spill-over [119]. This has been shown for other RNA viruses of which concentrations and detection rate increased in maternity roosts [120, 121]. The detection rate of KRV was significantly higher in juveniles compared to adults but the virus concentration remained constant during study time with a mean of $10^{6.44}$ viral RNA copies per gram tissue. For *E. helvum*, the birth season and the beginning of the wet season overlapped but no increase in virus concentration was observed. It seems as the virus life-cycle does not depend on the recruitment of susceptible animals.

Phylogenetically, KRV clustered within the supergroup of dimarhabdoviruses of which many were isolated from mammals and arthropods and their transmission cycles are often maintained by hematophagous arthropods [49, 122]. Considering the

observed seasonality, an arthropod transmission of KRV seems possible as an alternative option for transmission. However, mosquitoes from close proximity of the colony were tested without any evidence for the virus. The collection contained 10 different mosquito genera with varying numbers per genus. It remains possible that the tested sample size was not sufficient for the detection of KRV. On the other hand, the vesicular stomatitis virus (VSV) and Chandipura virus (CHPV), as members of the dimarhabdovirus supergroup, are transmitted by bites of phlebotomine sand flies [123, 124] which were not represented in the sampling. However, the vector for many dimarhabdoviruses is unknown, encouraging further studies on arthropods as possible vectors for the transmission and maintenance of KRV.

In this study the transmission of KRV to mammals was tested on cattle, goat, sheep and swine which are the main livestock species in Ghana. Neutralizing antibodies were detected in swine samples at low titres (1:10). A challenge when using serology as an indicator for past infections in different species, showing cross-species transmission, is that neutralizing antibody titers cannot be cross-compared between species in the same assay [125, 126]. Furthermore, the sample number for swine sera was low. On the other hand, *E. helvum* samples with low titers were also present and a very sensitive and specific PRNT assay was used. The question whether swine are infected accidentally and have low titers due to inadequate growth conditions for the virus or whether swine are able to host KRV and thus can develop high antibody tires should be answered in future studies with a broadened sample set. Thereby a direct detection of KRV in the organs and determination of the virus concentration is eligibly.

Neutralizing antibodies against KRV were also detected in one human sample. The sample originated from a patient of the Komfo Anokye Teaching Hospital in Kumasi with unknown clinical history. In cell culture experiments it was shown that KRV replicates in primate and humans cells to high titers, however several rhabdoviruses replicate to high titres in mammalian cell cultures [51] and it is difficult to draw conclusions towards possible hosts from this. In this study, serum samples of zoo workers were included, who are in close contact to the *E. helvum* colony as they live in the zoo during their working periods and are thus constantly exposed to urine, faeces

and parasites of the bats and also hunt them for control reasons. Therefore they are at high risk for acquisition of viruses from the colony. However, none of the zoo workers had neutralizing antibodies against KRV. It is therefore questionable if a transmission of KRV from bat to human is possible. Different transmission scenarios of KRV are imaginable, a direct transmission from bat to human and swine, a transmission from swine to human and vice versa, as well as an arthropod transmission between all three. Future studies should focus on increased sample sizes and study groups which are in close contact to swine.

Cross-neutralization among rhabdoviruses is untypical but possible, as the closely related Tibrogargan virus (TIBV) and Coastal plains virus (CPV) exhibit cross-neutralization [48]. They share an amino acid identity in their structural genes G and N of 56%, 67% respectively. KRV is closely related to MEBV and OIRV and clustered monophyletic with them. KCV formed a sister clade to the former and was isolated from an American *Myotis* bat, while MEBV and OIRV were isolated from Kenyan *Rhinolophus* bats [58, 127]. Although closely related it was shown that KCV and MEBV do not cross-neutralize [128]. Their amino acid identity in the N gene is 43% but only partial sequence information are available. On the contrary, KRV, MEBV and OIRV shared an amino acid identity in their partial N of 62-66%. Taking the amino acid identity of TIBV and CPV into consideration it is thus possible that KRV, MEBV and OIRV are cross-neutralizing. Exposure against these or other, as yet unidentified viruses might well explain the observed low-level neutralizing antibody activities in swine, putting any potential for KRV cross-species transmission into perspective. In future studies the focus should be on possible vectors for KRV, alternative transmission ways, the role of livestock and humans in the transmission cycle of KRV and the consequences of an infection.

4.3. Conclusions

4.3.1. Outcomes and future fields of research

E. helvum fruit bats carry a large diversity of henipa- and rubulaviruses, matching their migratory behaviour and dense community structure. These properties may promote virus acquisition, maintenance, and transmission to humans or livestock. Predictive life history traits could be used as indicators for the assessment of zoonotic risks associated with target species. However, it should be noted that the sampling in this and other virological investigations has been opportunistic with variations of the contribution of species to the sample determined by variation in trapping success. Unevenness in sampling is likely to be mirrored in the composition of viral gene database entries with uneven representation of viral taxa. A more even and phylogenetically representative sample of host taxa might enable assessments of ancestral virus-host associations and host change histories along viral evolutionary lineages. These in turn could identify actual cross-species viral transmissions.

Transmission of viruses to humans and livestock hosted by *E. helvum* may be possible as exemplified in this study for KRV. These investigations were facilitated through incidental isolation of the KRV primary virus from bats, enabling neutralization tests. Nevertheless, for a more systematic investigation of potential cross-species transmissions of other viruses hosted by *E. helvum*, neutralization assays based on recombinant proteins should be favored over lengthy and uncertain virus isolation trials. For the development of neutralization assays established viral pseudotyping systems such as the VSV- and HIV-1 vector backbones are available. These systems should be used in the future to investigate potential transmission *E. helvum* - associated henipa- and rubulaviruses as identified during this work, as members of those viral genera have been transmitted from bats to humans in other parts of the world, with sometimes severe pathogenicity. But also the implications of KRV on livestock and humans should be further explored in the future to exclude potential risks. In the past, individual cases of hemorrhagic fever in humans caused by so far unknown rhabdoviruses were reported. It can be assumed that the diversity of KRV-related

rhabdoviruses in *E. helvum* is considerable higher than known today. It is therefore of interest to assess rhabdovirus diversity and investigate their potential of transmission.

4.3.2. Biodiversity research with capacity building in source countries

Biological resources-rich countries in Africa are limited in their resources when it comes to basic research. A large part of this work has been conducted in Africa together with African co-operation partners, Principal investigators, and co-supervised staff and students. With some fundamental infrastructure such as laboratory rooms, basic equipment and continuous power supply in place, this study has proven that sensitive molecular biology methods such as RT-PCR assays can indeed be implemented in an African research setting. A problematic issue encountered during this work was the supply of reagents, consumables and other specific supply. Most of the materials used in modern molecular biology are not produced on the African continent and need to be imported. This creates challenges unknown to PhD students in the western world. Advanced payment of shipments are the rule when ordering from Africa. Custom procedures can be time-consuming and frustrating. The organization of transport to the final destination with maintenance of cooling chains is crucial and requires meticulous preparation and supervision. These difficulties will only change on the long run. The more projects are established in focused research settings such as in Kumasi/Ghana where this work was conducted, the better local suppliers can adjust to logistical demands and thereby reduce the organizational effort for the individual scientist. Among the greatest benefits in capacity building afforded by long-term projects in African research settings is the generation of an environment in which know-how and scientific ideas can be exchanged. African scientists can find themselves isolated from knowledge transfer that is normal in the western world and find it difficult to compete scientifically. The training of young ambitious scientists along with the provision of technical equipment will hopefully create a space for the development of an independent African scientific elite. Projects like this work provide a foundation to translate research-based methodology into more common applications such as diagnostic tests serving hospitals. For many of the locally relevant diseases diagnostic assays have never been developed, or are not applicable in local laboratories

without a research background. Scientific projects covered by research funds can substantially improve capacities and capabilities. However, the resources required for the provision of services in the medical field need to be provided by local health systems.

5. Summary

Bats are increasingly recognized as hosts of viruses which are significant for human and domestic health. However, the dynamics of these viruses in their natural hosts remain poorly elucidated. In this study, virus diversity and transmission was exemplified in two viral families present in the straw-coloured fruit bat (*E. helvum*).

Virus diversity and dynamics were investigated on the genera *Henipa*-and *Rubulavirus* of the family *Paramyxoviridae*. Phylogenetic analysis revealed a high diversity of both taxa in African fruit bats. The viruses were shared among other fruit bats to different extent and were detected in a variety of African countries. It was shown that the majority of these viruses were co-circulating during the sampling time of three years in a single bat colony from Kumasi, Ghana. Their potential to cross-species barriers was discussed based on their phylogentic relations, but transmission has to be investigated in more detail in the future. Viruses were predominantly detected in the spleen, but area of replication and transmission ways still need to be investigated.

In the frame of this study, a rhabdovirus named Kumasi rhabdovirus (KRV) was isolated and classified into the group of dimarhabdoviruses. Dimarhabdoviruses are often transmitted and maintained by arthropod vectors. KRV was detected in 5.1% of *E. helvum* from Kumasi. The virus was predominantly detected in the spleen and viral detection rates correlated with rain seasons suggesting an arthropod transmission. Serological analyse revealed 6.9% neutralizing antibodies in *E. helvum*. Cross-species transmission of KRV was shown for swine (5.4%) and humans (1.6%) and possible transmission ways were discussed.

Zusammenfassung

Fledermäuse werden zunehmend als Wirte für Viren die signifikant für Menschen und Vieh sind erkannt. Aber die Dynamik dieser Viren in ihren natürlichen Wirten ist schlecht verstanden. In dieser Studie, wurde die Virusdiversität und Übertragung an zwei Virus Familien aus dem Palmenflughund (*E .helvum*) dargestellt.

Die Virusdiversität und Dynamik wurde an den Genera *Henipa-* und *Rubulavirus* aus der Familie der *Paramyxoviridae* untersucht. Phylogenetische Analysen zeigten eine hohe Diversität beider Taxa in afrikanischen Flughunden. Die Viren wurden im unterschiedlichen Ausmaß unter den Flughundspezies geteilt und wurden in unterschiedlichen afrikanischen Ländern detektiert. Es wurde gezeigt, dass die Mehrheit der Viren in einer einzelnen Flughund Kolonie aus Kumasi, Ghana in dem Untersuchungszeitraum von drei Jahren zirkulieren. Ihr Potential Spezies Barrieren zu überwinden wurde diskutiert anhand von phylogenetischen Beziehungen aber das Übertragungspotential muss in der Zukunft weiter untersucht werden. Die Viren wurden hauptsächlich in der Milz detektiert aber der Ort der Replikation und die Übertragungswege sind noch ungeklärt.

Im Rahmen dieser Studie wurde ein Rhabdovirus, das Kumasi rhabdovirus (KRV) genannt wurde, isoliert und in die Gruppe der Dimarhabdoviren eingeordnet. Dimarhabdoviren werden häufig von arthropoden Vektoren erhalten und übertragen. KRV wurde in 5.1% der *E. helvum* aus Kumasi detektiert. Das Virus wurde hauptsächlich in der Milz gefunden und die Detektionsrate korreliert mit der Regenzeit, was auf eine arthropode Übertragung deutet. Serologische Analysen zeigten das 6.9% der *E. helvum* neutralisierende Antikörper besitzen. Die Übertragung auf andere Spezies wurde für Schweine (5.4%) und Menschen (1.6%) gezeigt und mögliche Übertragungswege diskutiert.

6. References

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7. Abbreviations

BASV	Bas-Congo virus
BEFV	Bovine ephemeral fever virus
cDNA	Complementary DNA
CHPV	Chandipura virus
CO_2	Carbon dioxide
Congo	Republic of the Congo
CPV	Coastal plains virus
CPE	Cytopathic effect
dATP	Desoxyadenosintriphosphat
dCTP	Desoxyribonukleosidtriphosphate
dGTP	Desoxyguanosintriphosphat
Dimarhabdovirus	Dipteran-mammal associated virus
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Desoxyribonukleosidtriphosphate
DRC	Democratic Republic of the Congo
dTTP	Desoxythymidintriphosphat
DURV	Durham virus
E. helvum	*Eidolon helvum*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf Serum
G	Glycoprotein
HCl	Chloric acid
ICTV	International Committee on Taxonomy of Viruses
IFA	Indirect immunofluorescence assay
IHNV	Infectious hematopoietic necrosis virus
KCV	Kern Canyon virus
KOTV	Kotonkan virus
KRV	Kumasi rhabdovirus
L	Large protein
LBV	Lagos bat virus
LNYV	Lettuce necrotic yellows virus
MARAV	Maraba virus
MEBV	Mount Elgon virus
MEM	Minimal essential medium
$MgCl_2$	Magnesiumchloride
$MgSO_4$	Magnesiumsulfate
MOI	Multiplicity of infection
MOUV	Moussa virus
MSPV	Malpais spring virus
N	Nucleocapsid
$NaHCO_3$	Natriumhydrogencarbonat
NaOH	Sodium hydroxide
NGAV	Ngaingan virus
NISV	Nishimuro virus

OD	Optical density
OIRV	Oita rhabdovirus virus
ORF	Overlapping reading frame
P	Phosphoprotein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PERV	Perinet virus
Pfa	Paraformaldehyde
PFRV	Pike fry virus
PFU	Plaque forming unit
PRNT	Plaque-reduction neutralization assay
PRV	Perch rhabdovirus
PYDV	Potato yellow dwarf virus
RABV	Rabies virus
RCA	Central African Republic
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SARS	Severe acute respiratory syndrome
SIGMAV	Drosophila melanogaster sigma virus,
TIBV	Tibrogargan virus
TMB	3,3',5,5'-Tetramethylbenzidine
Tris	Tris hydroxymethyl aminomethane
TRV	Tench virus
TUPV	Tupaia rhabdovirus
VSINV	Vesicular stomatitis Indiana virus
VSV	Vesiculo Stomatitis virus
WHO	The World Health Organization
WONV	Wongabel virus
β-propiolacton	Beta-propiolacton

Erklärung zur Dissertation

Ich erkläre, dass ich

- die Dissertation persönlich, selbstständig und ohne unerlaubte fremde Hilfe
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