Identification and characterisation of emerging viruses in free-ranging bats from sub-Saharan Africa

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Abstract

Emerging infectious diseases (EIDs) are an increasing threat to human health and a major challenge to global health care systems. Owing to their species richness, tropical regions, and especially sub-Saharan Africa, are predicted 'hotspots' for zoonotic EIDs, which stand for a vast majority of all EIDs. In these regions, monitoring bats might be a particularly fruitful strategy to detect emerging pathogens as their resistance to many viruses, their abundance, and their ability to fly point at them as major candidate reservoirs. The present study focused on free-ranging bats from tropical Africa, which were examined for viral infection using a broad range of techniques. This resulted in the discovery of viruses that were hitherto not known to infect bats. Most notably, the first indigenous bat hantavirus was found. In addition, the *Taï Forest reovirus*, a virus related to the human pathogenic *Colorado tick fever virus*, was isolated and its ability to infect human cells *in vitro* demonstrated. While the significance to human health of these divergent novel viruses remains to be investigated, the pathogenic potential of other bat viruses is already well established. This led us to examine the distributions of two major groups of human pathogenic viruses of zoonotic origin: filoviruses and paramyxoviruses (PMV). Antibodies directed against these viruses were detected in twelve bat species across West and Central Africa. As some of these species had not previously been associated with filoviruses, these findings substantially extend their recorded host and geographic range. Several aspects of bat PMV molecular epidemiology were investigated: Three bat species were shown to be infected with PMVs of considerably divergent origin. In depth analyses show that, despite the vast diversity of bat PMVs, cross-species transmission events from bats alone are not the major origin of presently circulating PMVs, which are rather exhibiting a equal frequency of primate-borne transmission events. The risk of transmission of PMVs from bats was further investigated by focusing on a plausible route of transmission to humans, namely bushmeat. Hunting, preparation, and consumption of meat from wildlife are indeed major risks for zoonotic pathogen transmission. This was investigated by examining bat bushmeat samples: PMVs were detected in more than one third of the corresponding animals. On the basis of these results one can assume a continuous exposition of the local population to these viruses, of which many were closely related to the highly pathogenic Hendra and Nipah viruses endemic in Australoasian flying foxes.

Local disease outbreaks caused by novel pathogens are regularly connected to increasing human intrusion into pristine ecosystems. International travel facilitates subsequent global pathogen spread, as seen during the 2003 SARS-pandemic. So far, it is not possible to foresee which pathogen will cross species barriers in the future. However, a better understanding of cross-species transmission processes, as developed in the present study, might help to design strategies to prevent or control future pandemics.

Zusammenfassung

Neuartige zoonotische Krankheitserreger stellen eine wachsende Bedrohung für den Menschen dar und sind eine der größten Herausforderungen des modernen globalen Gesundheitsmanagements. Tropische Regionen bergen aufgrund der dort vorhandenen Artenvielfalt ein besonderes Potential an unbekannten Erregern, und Fledertiere sind für eine ganze Reihe dieser zoonotischen Pathogene die natürlichen Wirte. Aufgrund ihrer Fähigkeit zu aktivem Flug und ihrer Resistenz gegen viele – für andere Säugetiere tödliche – Erreger kommt ihnen hierbei eine besondere Rolle zu. Im Rahmen der vorliegenden Studie wurden Proben von wildlebenden Fledertieren aus dem tropischen Afrika auf ein breites Spektrum an neuartigen Viren untersucht. Unter anderem wird das erste fledermauseigene Hantavirus beschrieben. Ein Teil der gewonnenen Fledertierproben wurde auf unbekannte Viren untersucht und ein Virusisolat im Rahmen der vorliegenden Arbeit beispielhaft weiter charakterisiert. Hierbei handelt es sich um ein neuartiges Virus aus der Familie der Reoviren, welches als *Taï Forest Reovirus* bezeichnet wurde. Es zeigt nächste Verwandtschaft zu dem humanpathogenen Colorado Zeckenfieber Virus und ist in der Lage *in vitro* humane Zellen zu infizieren. Während über das tatsächliche humanpathogene Potential dieser neuartigen Viren bisher keine verlässliche Aussage getroffen werden kann, ist die Relevanz anderer Fledertier-assoziierter Virusfamilien unstrittig: Filoviren und Paramyxoviren. Die Verbreitung dieser Viren in Afrikanischen Fledertieren wurde näher untersucht: In zwölf Fledertierarten konnten Antikörper gegen Ebola- oder Marburgvirus nachgewiesen werden; einige dieser Arten waren bisher nicht als Wirte für Filoviren bekannt. Antikörper gegen Marburgvirus wurden auch in Tieren aus Westafrika nachgewiesen, einer Region, aus der es bisher keine Berichte über das Vorkommen dieses Virus gab. Die Epidemiologie von Paramyxoviren (PMVs) wurde auf mehrere Aspekte hin untersucht: PMVs unterschiedlichen Ursprungs wurden in drei Fledertierarten detektiert. Tiefergehende phylogenetische Untersuchungen weisen darauf hin, dass trotz der extrem hohen Variabilität der detektierten Viren Fledertiere und Primaten gleichermaßen der Ursprung der meisten heute zirkulierenden PMVs zu sein scheinen. Ein besonderes Risiko zur Erregerübertragung von Wildtieren auf den Menschen besteht beim Umgang mit Wildfleisch. Um dies zu untersuchen, wurden Proben von zum Verzehr bestimmten Tieren gewonnen. In mehr als einem Drittel dieser Tiere konnten PMVs nachgewiesen werden, von denen einige nah verwandt mit den für den Menschen hochpathogenen Henipaviren sind. Aufgrund der hohen Infektionsrate der Tiere ist von einer andauernden Exposition der lokalen Bevölkerung auszugehen.

Das immer weitere Eindringen von Menschen in bisher unberührte Ökosysteme führt regelmäßig zu lokalen Krankheitsausbrüchen, die durch bisher unbekannte Erreger hervorgerufen werden. Deren Verbreitung wird durch den globalen Reiseverkehr zunehmend erleichtert, wie es schon bei der SARS-Pandemie 2003 beobachtet werden konnte. Da es bisher nicht möglich ist vorherzusehen welche Erreger zukünftig die Artenbarriere überschreiten werden, kann nur weitreichendes Wissen über die involvierten Virusfamilien und ein tiefgehendes Verständnis der zugrundeliegenden Übertragungsmechanismen dazu beitragen, Strategien zur Prävention und Kontrolle zukünftiger Pandemien zu entwickeln. Hierfür sind die vorliegenden Untersuchungen von größter Relevanz.

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Abbreviations

BEAST	Bayesian Ecological Analysis of Statistical Trends
BLAST	Basic Local Alignment Search Tool
BSL	biosecurity level
CPE	cytopathic effect
CTF	Colorado tick fever
CTFV	*Colorado tick fever virus*
dpi	days post infection
DRC	Democratic Republic of Congo
ds	double stranded
EBOV	*Ebola virus*
e.g.	exempli gratia
EID	emerging infectious diesease
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EmV	emerging virus
EPMV	Eidolon paramyxovirus
EV	*Eyach virus*
GOLA	Trans-boundary Peace Park
GP	glycoprotein
HCPS	hantavirus cardiopulmonary syndrome
HeV	*Hendra virus*
HFRS	haemorrhagic fever with renal syndrome
HIV	*Human immunodeficiency virus*
HRM	*Henipa-*, *Respiro-*, *Morbillivirus*
ICTV	International Committee on Taxonomy of Viruses
IUCN	International Union for the Conservation of Nature
MARV	*Marburg virus*
MGBV	*Magboi virus*
ML	maximum likelihood

MPI-EVAN	Max Planck-Institute for Evolutionary Anthropology
NCBI	National Center for Biotechnology Information
NiV	*Nipah virus*
NP	nucleoprotein
OD	optical density
ORF	open reading frame
p.i.	post infection
PAN-PCR	Particle-Associated Nucleic Acid PCR
PAR	*Paramyxovirinae*
PMV	paramyxovirus
PNOK	*Parc National d'Odzala-Kokoua*
PNT	*Parc National de Taï*
POD	peroxidase
pol	polymerase gene
PUTU	Putu Range
qPCR	quantitative PCR
RC	Republic of Congo
RCI	Republique Côte d'Ivoire
RESTV	*Reston ebolavirus*
RKI	Robert Koch-Institute
RPM	rounds per minute
SARS-CoV	*severe acute respiratory syndrome-coronavirus*
ss	single stranded
TFRV	*Taï Forest reovirus*
UNESCO	United Nations Educational, Scientific, and Cultural Organization
VHF	viral haemorrhagic fever
VP	viral protein
vs.	versus
WHO	World Health Organisation
ZEBOV	*Zaire ebolavirus*

1 Introduction

Emerging infectious diseases (EIDs) are an increasing threat to public health and global economies (Jones et al. 2008; Daszak et al. 2000). Jones et al. (2008) define EIDs as newly evolved pathogens (e.g. drug resistant strains), pathogens that have recently entered the human population for the first time, or pathogens that have been present in humans but increased in incidence. The majority of EIDs (∼60%) are zoonotic, that is they are transmitted from an animal reservoir host to humans. Of these, the vast majority (∼72%) originate in wildlife (Jones et al. 2008; Woolhouse & Gowtage-Sequeria 2005). Host species richness in wildlife was shown to be a significant predictor for EID emergence and, consistently, sub-Saharan Africa is one of the 'hotspots' for EID origin (Jones et al. 2008). There are contradictory reports about the actual proportion of various pathogen types on EIDs, but it is recognised that RNA viruses contribute substantially to that burden (Woolhouse & Gowtage-Sequeria 2005; Jones et al. 2008). Due to their high nucleotide substitution rate and lack of polymerase proof-reading ability, RNA viruses can comparably easily overcome species barriers and adapt to new hosts (Drake 1993; Moya et al. 2004). *Human immunodeficiency virus* (HIV), *severe acute respiratory syndrome coronavirus* (SARS-CoV), Influenza viruses (e.g. pandemic H5N1) and *Ebolavirus* are probably the best-known examples for zoonotic disease transmissions from wildlife to humans during the last decades.

The major cause of zoonotic EID events is anthropogenic interference: Environmental and behavioural changes such as human population expansion, agricultural encroachment, altered farming practises, introduction of new domestic species, bushmeat hunting, and wildlife trade are factors contributing to increased contact between humans, domestic animals, and wildlife. Deforestation leading to habitat fragmentation, habitat destruction, and roost disturbance forces animals into urban areas and thus in closer proximity to potential pathogen recipients like humans or livestock (Woolhouse & Gowtage-Sequeria 2005; Halpin et al. 2007; Jones et al. 2008). Eventually, these human intrusions lead to a loss of biodiversity which itself can elevate the risk of disease incidence and pathogen transmission when host species density increases, or when lost species are no or only suboptimal hosts for the pathogen (reviewed in Keesing et al. 2010).

During the last decades bats have increasingly been recognised as reservoir hosts for emerging viruses (EmV) that can cross species barriers and thus pose a threat for humans. Before the beginning of this study, a vast number of viruses had already been isolated from or associated with bats (Dobson 2005; Calisher et al. 2006; Wong et al. 2007). Despite the growing interest in bats as hosts for EmVs, knowledge on natural infections in these animals is scarce. Except for rabies virus, most studies on bat-borne viruses have been launched after human disease outbreaks

and focused on specific viruses in a given geographic area. No broad systematic study on bat-borne pathogens in African bat species has been available. Data from the present work will contribute to the description of pathogen diversity and dynamics in bat wildlife populations in tropical Africa. Linking this information to knowledge on host ecology might allow for more reliable estimations of transmission risks and could ultimately lead to the establishment of preventive measures before the next zoonotic disease transmission with potentially devastating effects occurs.

1.1 Aims

Aims of the present study are the identification and characterisation of novel, potentially zoonotic pathogens in free-ranging African bats of different species, focusing on virus families with known zoonotic potential.

A) Various bat samples originating from western and central Africa are screened using generic PCR systems and serological tests to

- identify host range, diversity and spatial distribution of paramyxoviruses
- investigate the spatial distribution of filoviruses with respect to the hypothesis of a wave-like spread of *Zaire ebolavirus* in Central Africa
- identify a possible distribution of hantaviruses in bats.

B) Cell cultures are inoculated with primary material obtained from captured animals to

- attempt virus isolation for viruses previously detected with PCR
- identify, isolate, and characterise novel and yet unknown viruses.

Generated sequence data will be the basis of phylogenetic analyses to classify detected viruses and to possibly identify candidates likely to cross species barriers. Comparing results obtained from different bat species from distinct geographic areas should contribute to the current knowledge on understanding of virus dynamics in wild bat populations. The results should help to estimate transmission risks, and to ultimately develop suitable methods to prevent future bat-derived disease transmission events or at least attenuate the consequences.

2 Background

Bats (Chiroptera) are among the oldest mammals of the world, having originated in the late Cretaceous Period ∼70 Million years ago (Meredith et al. 2011). They account for more than 1,200 species which make up 20% of all extant mammalian species and are distributed worldwide except for the polar and extreme desert areas (Wilson & Reeder 2005; Wong et al. 2007).
Bats feed on a variety of food sources including insects, small vertebrates, fruit, nectar, pollen, and blood, and many species use echolocation to locate and track their prey (Simmons 2005). These animals provide important services to ecosystems by pollinating plants and dispersing seeds (Kunz et al. 2011). Some insectivorous species eat up to 50% of their body mass, in insects every night (Brunet-Rossinni & Austad 2004), thereby also substantially contributing to control insect populations (Kunz et al. 2011). Despite their relatively low body mass bats have remarkably long life spans that can exceed 40 years (Brunet-Rossinni & Austad 2004; Podlutsky et al. 2005), but their most outstanding attribute is their ability to fly actively, which is unique among mammals.
Traditional taxonomy divides Chiroptera into microbats and frugivorous megabats (= Pteropodidae). Molecular data, however, support a different picture in which microbats are paraphyletic with the rhinolophoid families (Rhinolophidae, Hipposideridae, Megadermatidae, Rhinopomatidae, and Craseonycteridae) being more closely related to pteropodid bats than to the other microbats. Megabats together with the rhinolophoid bats comprise the clade Yinpterochiroptera (also called Pteropodiformes) while the remaining microbats comprise the clade Yangochiroptera (Almeida et al. 2011).
Like their feeding habits, roosting behaviour of different bat species differs widely. While some roost singly or in small groups others are highly gregarious and form colonies with thousands up to several million individuals. Shared roosts between species are common, especially among cave-dwelling animals. Roosting sites vary and can be tree canopies, hollow trees, or natural caverns. Some bat species are found in urban areas and use anthropogenic structures like disused huts, granaries, or roofs of houses as day roosts, which brings them in proximity to humans and livestock. Habitat ranges are also quite distinct between species. While some bats species remain rather local others migrate over large distances that can sum up to several thousand kilometres per year. Obviously, all these factors have an impact on pathogen maintenance in a given population and on transmission between species or mammalian orders. Short profiles of all bat species relevant for the present study are listed in the appendix A.1.

2.1 Bats as bushmeat

The use of bats as bushmeat throughout Africa and Asia has long been recognised (reviewed in Mickleburgh et al. 2009) and the consumption of pteropodid bats in south-east Asia, the Pacific Islands, and Madagascar is acknowledged as a threat for local bat populations (Mickleburgh et al. 2009; Jenkins & Racey 2008). Despite hunting being the primary cause for the IUCN (*International Union for Conservation of Nature*) near-threatened status of the straw-coloured fruit bat *Eidolon helvum*, the problem seems widely underestimated in mainland Africa. A study conducted in Ghana explains this underestimation mainly by the fact that bats are sold quickly and mostly outside the general markets, and reports *E. helvum* as the only bat species hunted and sold for bushmeat in Ghana (Kamins et al. 2011). Intensive contact through handling and consumption of wildlife species poses a huge potential for pathogen transmission, especially since animals are commonly handled without any protection (see figure 2.1). In general, information is scarce and concise is known yet about the risk of disease transmission through the use of bats as bushmeat.

Figure 2.1: *Eidolon helvum* prepared for selling on bushmeat markets. Photo by K. Nowak.

2.2 The role of bats in the epidemiology of EIDs

Some of the life history traits mentioned above render bats particularly suitable hosts for pathogens. Indeed, they are known reservoirs for a number of emerging pathogens, among these some of the most deadly viruses, like *Rabies*-, *Ebola*-, *Hendra*, and *Nipah virus* and the SARS-CoV (Calisher et al. 2006). Whilst these viruses usually kill their mammalian hosts they apparently do not harm bats, probably due to the lack of a gene family involved in the innate immune response (Zhang et al. 2013). Despite their vast number bats do not harbour a disproportionate number of zoonotic EIDs (Dobson 2005; Woolhouse & Gowtage-Sequeria 2005), but a major concern

is that their ability to fly and their longevity enable them to spread pathogens not only over large distances, but also during a prolonged period of time. The potential of different species to spread pathogens is obviously influenced by specific feeding and roosting behaviour, migratory behaviour, as well as the choice of roosting sites. Pathogen transmission between individuals commonly occurs directly via body fluids or indirectly through vectors (e.g. arthropods) and is favoured where animals live in high densities (e.g. huge colonies). Another scenario is transmission via intermediate hosts, which can provide a bridge between different host species that would otherwise only have little contact. Examples are the spread of *Nipah virus* and of the SARS-CoV, where pigs and palm civets, respectively, served as amplifying intermediate hosts between bats and humans (see following section; Field et al. 2001; Pepin et al. 2010).

2.3 Emerging viruses associated with bats

The present study focuses on virus families with known zoonotic potential. While two virus families (*Paramyxoviridae* and *Filoviridae*) have previously been associated with bats, the present work describes the first indigenous hanta- and reovirus in African bats. This section provides a short overview about those viruses and virus families relevant for the present study.

2.3.1 *Paramyxoviridae*

The family *Paramyxoviridae* is a member of the *Mononegavirales* and comprises the two subfamilies *Paramyxovirinae* and *Pneumovirinae*. These include a great variety of different viruses, many of them pathogenic for humans or livestock, for example *Mumps virus*, *Measles virus*, *Rinderpest virus*, and various parainfluenza and distemper viruses. Paramyxoviruses are enveloped viruses with a single-stranded RNA genome in negative orientation. Besides a great variety of mammals, reptiles and birds are known hosts of these viruses.
Highest concern has been raised by the relatively new genus *Henipavirus* in the recent past. *Hendra virus* (HeV), was discovered in 1994 when killing 15 horses and 2 humans in two spatially distinct disease outbreaks and has since re-emerged multiple times (Murray et al. 1995; Marsh et al. 2012). The second virus of the genus, *Nipah virus* (NiV), was identified in 1999 during a huge disease outbreak in piggeries in Malaysia, resulting in the culling of more than one million pigs and the death of more than 100 humans (Chua et al. 1999; Chua 2000). Since then, NiV has evolved into a major health problem in Bangladesh (Luby et al. 2009; Stone 2011; Clayton et al. 2012). Both viruses were traced back to Indo-Pacific bats of the genus *Pteropus* as reservoir host (Field et al. 2001). As typical for reservoir host species, pteropodid bats do not show clinical symptoms upon experimental infection (Williamson et al. 1998; Halpin et al. 2011; Middleton et al. 2007). The distribution of viruses is limited by the distribution of their host species and pteropodid bats are only endemic in Australia, Asia and islands east of Africa, but not mainland Africa. Hence, henipavirus distribution was believed to be restricted, until antibodies and henipavirus-related RNA were found in *Eidolon helvum* from Ghana, West Africa, and in *Eidolon dupreanum* from Madagascar (Hayman et al. 2008; Drexler et al. 2009; Iehlé et al. 2007). Today, various paramyxoviruses have been described in different bat species on all continents (Henderson et al.

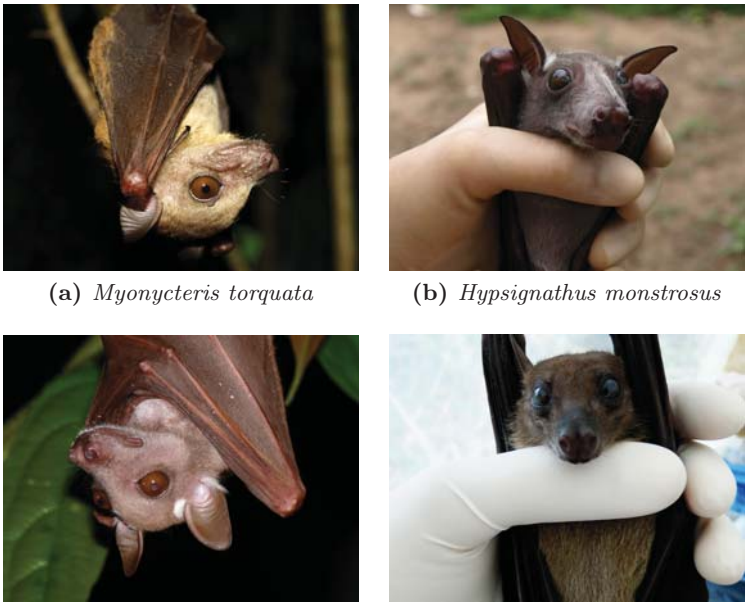
1995; Chua et al. 2002; Lau et al. 2010; Kurth et al. 2012; Wilkinson et al. 2012; Baker et al. 2012; Drexler et al. 2012), but nothing is yet known about the pathogenic potential of these viruses in humans.

2.3.2 *Filoviridae*

Ebola- and *Marburgvirus* are the only members of the family *Filoviridae* (*Mononegavirales*), and are enveloped viruses with a linear, non-segmented, single stranded negative RNA genome. In humans, great apes, and most other mammals, filoviruses cause viral haemorrhagic fever (VHF) with fatality rates ranging from 53% to 90% in large ($>$ 100 infected individuals) outbreaks (Leroy et al. 2011).
The genus *Marburgvirus* (MARV) comprises only one species, *Lake Victoria marburgvirus* or *Marburg marburgvirus*. Out of five lineages, one differs remarkably by up to 21% (compared to $<$ 8%) and hence is considered as a second virus, *Ravn virus*, in the same species. For *Ebolavirus* (EBOV) five species have been described so far, that differ by 32–42% on nucleotide level: *Sudan ebolavirus*, *Zaire ebolavirus* (ZEBOV), *Taï Forest ebolavirus*, *Reston ebolavirus* (RESTV) and *Bundibugyo ebolavirus* (Kuhn et al. 2010; Barrette et al. 2009). Of these, only RESTV is not endemic in Africa, but the Philippines (Jahrling et al. 1990), and the only known filovirus most likely not causing disease in humans (Miranda & Miranda 2011). A novel filovirus, *Lloviu virus* has been detected in Schreiber's long-fingered bat (*Miniopterus schreibersii*) in Cueva del Lloviu, Spain (Negredo et al. 2011). Since it differs equally from EBOV and MARV (51% and 56%, respectively), the creation of a new genus, *Cuevavirus*, has been proposed (Kuhn et al. 2010).
Marburgvirus was first discovered in 1967 when lab workers handling imported African green monkeys (*Cercopithecus aethiops*) fell sick in Marburg, Germany, and Belgrade, Serbia (former Yugoslavia). Since then only sporadic cases have been reported from Africa until large outbreaks occurred 1998–2000 in DRC and 2004–2005 in Angola (reviewed in Leroy et al. 2011 and Amman et al. 2012). Latest reports stem from a MARV-caused VHF outbreak in Uganda in 2012 (WHO 2012). *Ebolavirus* was first described in 1976 in Yambuku, on the border between DRC and Sudan, and has since frequently led to outbreaks in humans and great apes in Africa (reviewed in Groseth et al. 2007; Leroy et al. 2011), most recently in Uganda preceding the 2012 MARV-related VHF outbreak (WHO 2013). Besides commercial hunting and expanding logging activities, *Ebolavirus* is considered a major reason for the severe decline in gorilla and chimpanzee populations in the last decades (Bermejo et al. 2006; Walsh et al. 2003; Formenty et al. 1999). During the past decade, a remarkably high number of VHF outbreaks caused by ZEBOV has been recorded in Central Africa, especially at the border of Gabon and the Republic of Congo. According to Walsh et al. (2005) these outbreaks were caused by virus species descendant from the Yambuku-virus, which is predicted to spread in a wave-like manner through the region (see section 4.1.1; Walsh et al. 2005).

Fruit bats are supposed to be the natural reservoir for filoviruses: *Ebolavirus* RNA has been amplified from liver and spleen tissues of three African fruit bat species: *Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquata* (Leroy et al. 2005; see figure 2.2 a-d). Antibodies

have been detected in additional species: *Micropteropus pusillus*, *Rousettus aegyptiacus*, and *Mops condylurus* in Africa, and in *R. amplexicaudatus* and *R. leschenaulti* in the Philippines and in Bangladeh, respectively (Pourrut et al. 2007, 2009; Taniguchi et al. 2011; Olival et al. 2013). Yet, no virus has been isolated from any free-ranging bat. The situation is more obvious for MARV: There is increasing evidence for the cave-dwelling fruit bat *R. aegyptiacus* (see figure 2.2 d) being the primary reservoir host (Amman et al. 2012), even though MARV-specific antibodies have also been detected in other bat species (*E. franqueti*, *H. monstrosus*, *M. pusillus*, *Rhinolophus eloquens*, and *Miniopterus inflatus*), but with lower frequency (Swanepoel et al. 2007; Pourrut et al. 2009; Towner et al. 2009). Various MARV lineages have been isolated from *R. aegyptiacus* (Towner et al. 2009) and human outbreaks have frequently been connected to cave-visits of index patients (Bausch et al. 2006; Amman et al. 2012). Just recently, Amman and colleagues showed that infection of older *R. aegyptiacus* juveniles peaks during the biannual birthing season, which correlates temporally with the majority (85%) of human infections suspected to be the result of discrete spill-over events direct from nature (Amman et al. 2012).



(a) *Myonycteris torquata* **(b)** *Hypsignathus monstrosus*

(c) *Epomops* sp. **(d)** *Rousettus aegyptiacus*

Figure 2.2: Filovirus host species. Photos (c) and (d) by K. Nowak.

Filoviruses can be transmitted between humans via body fluids, including blood, saliva, stool, semen, breast milk, and tears (Bausch et al. 2007). However, transmission from wildlife to humans commonly occurs accidently through contact with deceased carcasses (Rouquet et al. 2005). Direct bat to human transmission seems to be common for *Marburgvirus* (Amman et al. 2012) but only one human Ebola VHF outbreak has yet been traced back directly to fruit bats (Leroy et al. 2009). In contrast to severe diseases caused by filoviruses in most mammals and consistent with the assumption of bats being the primary reservoir, infection in these animals seems to be asymptomatic (Swanepoel et al. 1996; Towner et al. 2009; Kuzmin et al. 2010).

2.3.3 *Reoviridae*

The family *Reoviridae* (respiratory, enteric, orphan virus) harbours non-enveloped viruses with a segmented double-stranded RNA genome. They are currently divided into two subfamilies, the *Sedoreovirinae* comprising six, and the *Spinareovirinae* comprising nine genera. To date, a number of bat-borne reoviruses have been described, all of which belong to the genus *Orthoreovirus* in the latter subfamily (Gard & Compans 1970; Pritchard et al. 2006; Chua et al. 2007; Cheng et al. 2009; Du et al. 2010; Thalmann et al. 2010; Lelli et al. 2012; Kohl et al. 2012). Some of these, namely *Melaka virus*, *Kampar virus*, and *Pteropine Reovirus 7S*, have been isolated from humans suffering from acute respiratory illness in Malaysia, and showed capability of human to human transmission (Chua et al. 2007, 2008, 2011). However, they have only been epidemiologically linked to fruit bats yet.

In general, reoviruses cause rather mild diseases in humans. One genus is outstanding in this respect in that its members can cause more severe clinical diseases in humans: The genus *Coltivirus* is found within the subfamily *Spinareovirinae* and comprises two species only: *Colorado tick fever virus* (CTFV) and *Eyach virus* (EV). CTFV is the etiologic agent of a febrile human disease, Colorado tick fever (CTF), which occurs in the Rocky Mountains in the Western United States (US) and Canada (Florio et al. 1946; Attoui et al. 2000, 2002). CTF was claimed to be the most common arthropod-transmitted, clinically overt, viral human disease in the US (Emmons 1988; Attoui et al. 2000). It is rarely fatal but can cause severe complications like encephalitis, haemorrhage, or pericarditis, especially in children (Kapikian & Shope 1996). However, death is rare. The antigenically related EV was isolated in Germany in 1976 and has only indirectly been associated with human neurological disease by serological evidence (Rehse-Küpper et al. 1976; Málková et al. 1980). No related viruses have yet been reported from Africa.

The natural animal reservoirs of coltiviruses are small mammals like rodents and lagomorphs and transmission to humans occurs by ticks of the family Ixodidae (Eklund et al. 1958; Emmons 1988; Attoui et al. 2002). Although CTFV has been isolated from various tick species, the principle vector is the Rocky mountain wood tick (*Dermacentor andersoni*) whose distribution limits the distribution of the virus and which is also the only known proven vector for humans (Emmons 1988). The European EV has been isolated from Castor bean ticks (*Ixodes ricinus*) and the Rabbit tick (*Ixodes ventalloi*) in Germany and France, respectively (Rehse-Küpper et al. 1976; Chastel et al. 1984). Despite various hypotheses about the vector, it is recognised that EV originated from CTFV and was introduced into Europe most likely during the past century (Attoui et al. 2002; Hubálek & Rudolf 2012).

2.3.4 Hantavirus

Hantaviruses (family *Bunyaviridae*) are enveloped viruses with a segmented (three segments: S, small; M, medium; L, large) single stranded RNA genome in negative orientation. The prototypic hantavirus *Hantaan virus*, was first described in 1978 by Lee et al. (1978) but the associated disease, Korean haemorrhagic fever, had already been observed during the Korean War (1950-1953). So far, hantaviruses have been described on all continents except Australia and until

today 23 virus species are listed by the International Committee on Taxonomy of Viruses (ICTV; King et al. 2012). Hantaviruses are special among bunyaviruses by being the only representatives not transmitted by arthropods. Instead, infection occurs via exposure to virus-containing saliva and excreta (Root et al. 2004; Hinson et al. 2004; Glass et al. 1988; Bagamian et al. 2012). The natural reservoirs of hantaviruses are small mammals like rodents (Rodentia), moles, and shrews (Soricomorpha), in which they cause no or only mild disease (reviewed in Jonsson et al. 2010 and Schönrich et al. 2008, Luis et al. 2012). Besides rodents and soricomorphs, hantavirus antigen and nucleic acid was also found in lung and kidney tissues of bats (Chiroptera) from the genera *Eptesicus* and *Rhinolophus* in South Korea. However, nucleotide sequencing showed the presence of prototypical *Hantaan virus* indicating a spillover infection or laboratory contamination (Kim et al. 1994; Jung & Kim 1995).

Hantaviruses typically show strong association with their host species, but it is argued whether they co-diverged with their hosts (Kang et al. 2009, 2011) or whether this is a result of preferential host switching and local adaptation (Ramsden et al. 2009). A schematic overview about the different host species is given in figure 2.3. When transmitted to humans, commonly via inhalation of contaminated excreta (Kallio et al. 2006; Schönrich et al. 2008; Krüger et al. 2011), rodent-borne hantaviruses can cause life-threatening diseases: hantavirus cardiopulmonary syndrome (HCPS) in the Americas and haemorrhagic fever with renal syndrome (HFRS) in Asia and Europe (reviewed in Jonsson et al. 2010). Given the close relationships of both, virus and host, those diseases are surprisingly distinct. With the exception of *Andes virus* (ANDV), no human-to-human transmission has yet been reported (Wells et al. 1997; Padula et al. 1998; Martinez et al. 2005; Ferres et al. 2007), and so far there is no evidence for vertical transmission in rodents or humans (Pai et al. 1999; Borucki et al. 2000; Botten et al. 2002; Taruishi et al. 2008).

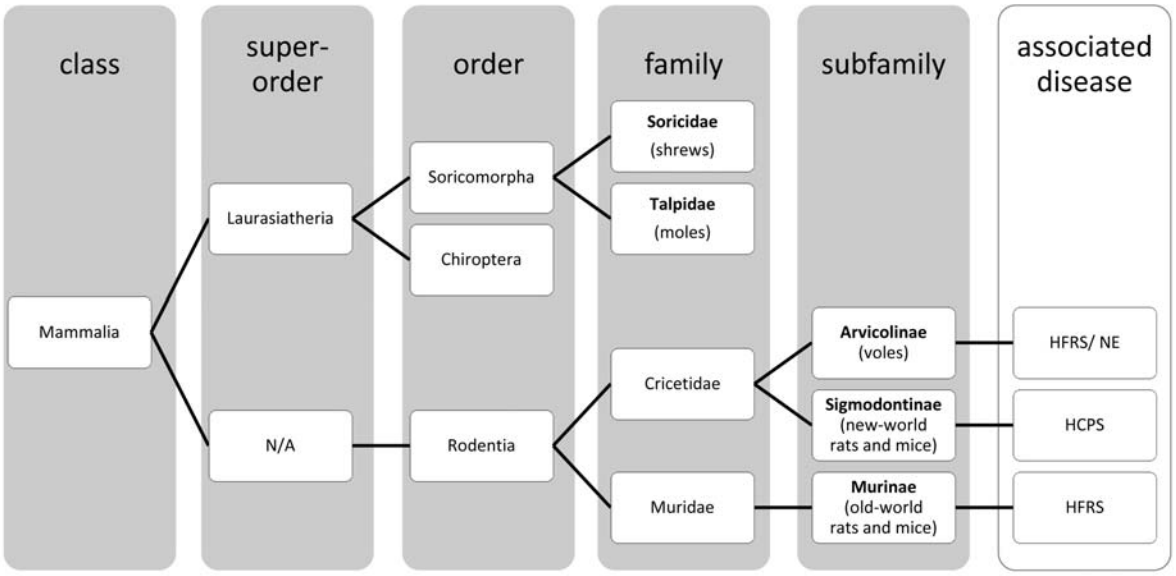


Figure 2.3: Schematic overview of hantavirus hosts known to date and diseases in humans caused by associated viruses. Taxonomic division of hosts is only given as referred to in the text. HFRS, haemorrhagic fever with renal syndrome; NE, nephropatia epidemica; HCPS, hantavirus cardiopulmonary syndrome; N/A, not applicable.

Virus-origin in respect to their animal host seems to have an influence on pathogenicity in humans, but to date, nothing is known about the pathogenic potential of shrew- and mole-borne hantaviruses in humans (Krüger et al. 2011). Among the rodent-borne viruses those harboured by voles (Cricetidae, subfamily Arvicolinae) have no or much less severe pathogenic potential in humans (Jonsson et al. 2010) compared to those of the subfamily Sigmodontinae (new world mice and rats) or long-tailed mice (family Muridae). While the latter are causing HCPS and HFRS, respectively, *Puumala virus* (PUUV) is the only human pathogenic vole-associated virus causing nephropatia epidemica (NE), a mild form of HFRS. Pathogenicity of different hantaviruses also seems to be coupled to receptor use. Viruses showing no or low pathogenic potential use β1-integrins to enter human cells in vitro while pathogenic viruses use β3-integrins (Gavrilovskaya et al. 1998, 2008; Schönrich et al. 2008; Song et al. 2007). *Thottapalayam virus* (TPMV), the prototypic shrew-borne hantavirus, also uses β1-integrins (Song et al. 2007), but nothing further is known about receptor use of other soricomorph-borne hantaviruses.

3 Material

3.1 Equipment

Analytical balance	Sartorius AG, Göttingen
Beckmann ultracentrifuge (rotor SW 32 Ti)	Beckmann Coulter Inc., Krefeld
Biological Safety cabinet HeraSafe	Thermo Fisher Scientific Inc., Rockfort, IL, USA
Cell disruptor (FastPrep®-24 Instrument)	MP Biomedicals, Heidelberg
Centrifuge EP 5417C	Eppendorf AG, Hamburg
CO2 Incubator C200	Labotect GmbH, Göttingen
ELISA-reader (Sunrise^{TM})	Tecan Deutschland GmbH, Crailsheim
ELISA-washer (Columbus Pro^{TM})	Tecan Deutschland GmbH, Crailsheim
Fast Blot B34	Biometra GmbH, Göttingen
FlexCycler	Biozym Scientific GmbH, Hessisch Oldendorf
Gel documentation system	PHASE Geselleschaft für Phorese, Analytik und Separation mbH, Lübeck
Gel electrophoresis chamber	neoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg
Incubator Heraeus B6060	Thermo Fisher Scientific GmbH, Dreieich
Light microscope (Motic AE20)	Ehlert & Partner GbR, Niederkassel-Rheidt
Mastercycler ep Gradient	Eppendorf AG, Hamburg
Microwaves	SB-Großhandels GmbH, Quelle Gruppe, Nürnberg
Mini PROTEAN II	Bio-Rad Laboratories GmbH, München
Nanodrop® ND-1000	PEQLAB Biotechnologie GmbH, Erlangen
Neubauer counting chamber	Carl Roth® GmbH & Co KG, Karlsruhe
New Brunswick innova 4200 Incubator-Shaker	Eppendorf AG, Hamburg
Pipettes	Eppendorf AG, Hamburg
Pipetus®-akku	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt
Power supply (Consort EV2.31)	Sigma-Aldrich Chemie GmbH, Steinheim
PowerPac Basic Power Supply	Bio-Rad Laboratories GmbH, München
Precision balance	Sartorius AG, Göttingen
Refrigerated SIGMA 2-16K Centrifuge	Sigma Laborzentrifugen GmbH, Osterode am Harz
Sequencer (ABI Prism 3130xl Genetic Analyzer)	Life Technologies^{TM} GmbH, Darmstadt
Shaker (IKA®HS 260 basic)	Sigma-Aldrich Chemie GmbH, Steinheim
Taqman Stratagene Mx3000P	Agilent Techologies, Inc.; Santa Clara, CA, USA
Thermomixer compact	Eppendorf AG, Hamburg
Vortexer (REAX top)	Heidolph Instruments GmbH & Co.KG, Schwabach
Water bath	GFL Gesellschaft für Labortechnik GmbH, Burgwedel

3.2 Kits

Ambion® TURBO DNA-free™	Life Technologies™ GmbH, Darmstadt
Applied Biosystems® BigDye® Terminator v3.1 Cycle Sequencing Kit	Life Technologies™ GmbH, Darmstadt
ExoSAP-IT® For PCR Product Clean-Up	Affymetrix® Inc., Santa Clara, CA, USA
GeneMATRIX Stool DNA Purification Kit	Roboklon, Berlin
Invitrogen™ 5' RACE System for Rapid Amplification of cDNA Ends	Life Technologies™ GmbH, Darmstadt
Invitrogen™ Platinum® Taq DNA Polymerase	Life Technologies™ GmbH, Darmstadt
Invitrogen™ SuperScript® II Reverse Transcriptase	Life Technologies™ GmbH, Darmstadt
Invitrogen™ TOPO® TA Cloning® Kit for Sequencing with One Shot® TOP10 Chemically Competent E. coli	Life Technologies™ GmbH, Darmstadt
JETQUICK Gel Extraction Spin Kit	Genomed GmbH, Löhne
NucleoSpin® RNA II	Macherey-Nagel GmbH & Co. KG, Düren
Plasmid Mini Prep Kit	Macherey-Nagel GmbH & Co. KG, Düren
QIAamp Viral RNA Mini Kit	QIAGEN GmbH, Hilden
SuperSignal® West Dura Extendet Duration Substrate	Thermo Fisher Scientific Inc., Rockfort, IL, USA
SureBlue™ TMB Microwell Peroxidase Substrate	Kirkegaard & Perry Laboratories, Inc.; Gathersburg, MD, USA
Universal RiboClone cDNA Systhesis System	Promega GmbH, Mannheim

3.3 Cell lines

C6/36 (*Aedes albopictus* clone c6/36, whole larvae):	ATCC®-number: CRL-1660™
Hep2 (HeLa derivative, Human cerix carcinoma):	ECACC-number: 86030501
MDCK II (Canine Cocker Spaniel Kidney):	ECACC-number: 00062107
MRC-5 (Human lung fibroblasts):	ATCC®-number: CCL-171™
R05T (*Rousettus aegyptiacus* fetal head tissue):	Jordan *et al.*, 2009
RK13 (Rabbit kidney):	ECACC-number: 00021715
VeroE6 (*Cercopithecus aethiops* Vero C1008, kidney):	ATCC®-number: CRL-1586™

ATCC® = American Type Culture Collection
ECACC = European Collection of Cell Cultures

3.4 Consumables

ABgene PCR Plates	Thermo Fisher Scientific Inc., Rockfort, IL, USA
Cell culture flasks (25, 75, 175 cm^2)	eBioscience, Frankfurt
Centrifuge tube (15 ml, 50 ml)	neoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg
Clear Seal Diamond, ABgene	Thermo Fisher Scientific Inc., Rockfort, IL, USA
Cotton swabs (different sizes, sterile)	Heinz Herenz Medizinalbedarf GmbH, Hamburg
Cryotubes (1.2 ml)	Carl Roth® GmbH & Co. KG, Karlsruhe
Embryonated chicken eggs	Valo Biomedia GmbH, Osterholz-Scharmbeck
Filter paper (903)	GE Healthcare, Buckinghamshire, UK
Filters (0.22 µm, 0.45 µm)	Merck Millipore, Billerica, MA, USA
Forceps (disposable)	Carl Roth® GmbH & Co. KG, Karlsruhe
Medical X-Ray Screen Film blue	Agfa HealthCare NV, Mortsel, Belgium
Micro tubes (2 ml)	SARSTEDT AG & Co., Nümbrecht
Micropipette tips	nerbe plus GmbH, Winsen (Luhe)
Nitrocellulose membrane	Thermo Fisher Scientific GmbH, Dreieich
Nunc MaxiSorp® flat-bottom 96 well plate	eBioscience, Frankfurt
Parafilm® M	BRAND GMBH & CO KG, Wertheim
Precellys ceramic beads (Ø 1.4 mm)	PEQLAB Biotechnologie GmbH, Erlangen
Reaction tubes (0.5 ml)	Carl Roth® GmbH & Co. KG, Karlsruhe
Reaction tubes (1.5 ml, 2 ml)	SARSTEDT AG & Co., Nümbrecht
Scapels (disposable)	Carl Roth® GmbH & Co. KG, Karlsruhe
Surgical gloves (Biogel® Diagnostic™)	Mölnlycke Health Care GmbH, Erkrath
TYVEK® body suits	Carl Roth GmbH & Co. KG, Karlsruhe
Ultra-Clear™ Centrifuge tubes	Beckman Coulter GmbH, Krefeld
Whatman™ paper (GB003)	GE Healthcare, Buckinghamshire, UK

3.5 Chemicals

Invitrogen™ 1 kb DNA ladder	Life Technologies™ GmbH, Darmstadt
Invitrogen™ 100 bp DNA ladder	Life Technologies™ GmbH, Darmstadt
2-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Steinheim
30% NF-Acrylamid/Bis-solution (29:1)	Carl Roth® GmbH & Co. KG, Karlsruhe
5-bromo-4-chloro-3-indolyl-b-D-Galactopyranoside (X-Gal)	Carl Roth® GmbH & Co. KG, Karlsruhe
Acetic acid ($C_2H_4O_2$)	Carl Roth® GmbH & Co. KG, Karlsruhe
Agar	BD, Heidelberg
Ambion® Nuclease-free water	Life Technologies™ GmbH, Darmstadt
Ambion® RNAlater®	Life Technologies™ GmbH, Darmstadt
Ammonium persulphate (APS)	Carl Roth® GmbH & Co. KG, Karlsruhe
Ampicillin Sodium Salt	Carl Roth® GmbH & Co. KG, Karlsruhe
Bacto™ Tryptone	BD, Heidelberg
Bacto™ yeast extract	BD, Heidelberg
Blue/Orange Loading Dye, 6X	Promega GmbH, Mannheim
Bromphenol blue	Carl Roth® GmbH & Co. KG, Karlsruhe
carrier RNA	QIAGEN GmbH, Hilden
Diethylether	Carl Roth® GmbH & Co. KG, Karlsruhe
Donkey anti-Goat IgG (H&L)-POD	DIANOVA GmbH, Hamburg
Ethanol	Carl Roth® GmbH & Co. KG, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth® GmbH & Co. KG, Karlsruhe
Fetal calf serum (FCS)	PAN-Biotech GmbH, Aidenbach
GelRedTM Nucleic Acid Gel Stain	VWR International GmbH, Darmstadt
Gibco® Dulbecco's Modified Eagle Medium (D-MEM)	Life Technologies™ GmbH, Darmstadt
Gibco® Leibovitz's L-15 medium	Life Technologies™ GmbH, Darmstadt
Gibco® Typsine-EDTA	Life Technologies™ GmbH, Darmstadt
Glutaraldehyde (GA)	Carl Roth® GmbH & Co. KG, Karlsruhe
Glycerine	Carl Roth® GmbH & Co. KG, Karlsruhe
Glycin	Carl Roth® GmbH & Co. KG, Karlsruhe
Goat anti-Bat IgG-h+l-POD	Bethyl Laboratories, Inc.; Montgomery, TX
Invitrogen™ Deoxynucleoside triphosphate (dNTP)	Life Technologies™ GmbH, Darmstadt
Invitrogen™ Desoxyuridine triphosphate (dUTP)	Life Technologies™ GmbH, Darmstadt
Isopropyl-ß-D-thiogalactopyranoside (IPTG)	Carl Roth® GmbH & Co. KG, Karlsruhe
Ketamine (50 mg/ ml)	WDT eG, Garbsen
L-Glutamine	Biochrom AG, Berlin
Methanol	Carl Roth® GmbH & Co. KG, Karlsruhe
PageRuler™ Plus Prestained Protein Ladder	Fermentas GmbH, St. Leon-Rot
Penicillin/Streptomycin	Biochrom AG, Berlin
peqGOLD Universal Agarose	PEQLAB Biotechnologie GmbH, Erlangen
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium chloride (KCl)	Sigma-Aldrich Chemie GmbH, Steinheim
Primers and probes	TIB MOLBIOL Syntheselabor GmbH, Berlin
Rompun® (Xylazine) 2%	Bayer AG, Leverkusen
Saccharose	Carl Roth® GmbH & Co. KG, Karlsruhe
Skimmed milk powder (Vival Naturaflor)	Töpfer GmbH, Dietmannsried
Sodium chloride	Carl Roth® GmbH & Co. KG, Karlsruhe
Sodium dodecyl sulfate (SDS)	Carl Roth® GmbH & Co. KG, Karlsruhe
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich Chemie GmbH, Steinheim
Tetramethylethylenediamine (TEMED)	Carl Roth® GmbH & Co. KG, Karlsruhe
Tris	Carl Roth® GmbH & Co. KG, Karlsruhe
TRIS-hydrochlorid (Tris-HCl)	Carl Roth® GmbH & Co. KG, Karlsruhe
Tween 20	Carl Roth® GmbH & Co. KG, Karlsruhe

3.6 Solutions

TAE-buffer (50x), pH 8	
Tris	242.28 g
EDTA	18.61 g
acetic acid	60 ml
ad ddH_2O	1000 ml

Luria-Bertani (LB) agar, pH 7	
Bacto-typtone	10 g
Bacto yeast extract	5 g
NaCl	10 g
agar	15 g
Ampicillin	10 g
ad ddH_2O	1000 ml
X-Gal	200 mg/ml
IPTG	0.1 M
ad ddH_2O	1000 ml

LB-medium, pH 7	
Bacto-typtone	10 g
Bacto yeast extract	5 g
NaCl	10 g
Ampicillin	10 g
ad ddH_2O	1000 ml

Phosphat-buffered saline (PBS), pH 7.2	
KCl	0.2 g
(Na_2HPO_4)	1.44 g
(KH_2PO_4)	0.24 g
ad ddH_2O	1000 ml

Western blot

1.5 M Tris; pH 8.8	
Tris	90.86 g
ddH_2O	400 ml
adjust pH with HCl	
ad ddH_2O	500 ml

1 M Tris; pH 6.8	
Tris	60.57 g
ddH_2O	400 ml
adjust pH with HCl (ca 80 ml/30%)	

10x SDS-buffer	
Tris	30.29 g
Glyceine	144.13 g
SDS	1% (w/v)
ad ddH_2O	1000 ml

2x Laemmli-buffer	
SDS (20%)	2 ml
ddH_2O	2 ml
Glycerine	2 ml
Tris-HCl (0.5 M)	2.5 ml
Bromphenolblau (0.1% (w/v))	1 ml

Transfer-buffer	
Tris	3.03 g
Glycine	14.4 g
Methanol	100 ml
ad ddH_2O	1000 ml

3.7 Software

Adobe Creative Suite (Photoshop CS5, Illustrator CS5.1)	Adobe Systems Software Ireland Limited, Dublin, Ireland
Beast Package (including Tree Annotator, LogCombiner)	Drummond & Rambaut (2007) http://beast.bio.ed.ac.uk/
DNASTAR Lasergene 10 Core Suite (SeqMan Pro)	DNAStar, Inc.; Madison, WI, USA
FaBox v1.4	Villesen (2007) http://birc.au.dk/software/fabox
Fig Tree v1.4.0	http://tree.bio.ed.ac.uk/software/figtree/
Geneious Pro v5.5.7	Biomatters Ltd., Auckland, New Zealand
Graph Pad Prism 5	GraphPad Software, Inc.; La Jolla, CA, USA
jModeltest 0.1.1	Posada (2008) http://darwin.uvigo.es/
Magellan^{TM} - Data Analysis Software	Tecan Deutschland GmbH, Crailsheim
MEGA5	Tamura et al. (2011) http://www.megasoftware.net/
Mesquite v2.75	Maddison & Maddison (2011) http://mesquiteproject.org
Microsoft Office 2010	Microsoft, Redmont, WA, USA
MxPro 4.1	Agilent Techologies, Inc.; Santa Clara, CA, USA
NCBI ORF finder	NCBI, Bethesda, MD, USA
Patristic	Fourment & Gibbs (2006) http://www.bioinformatics.org/patristic/
R	http://www.r-project.org/
SeaView version 4	Gouy et al. (2010) http://pbil.univ-lyon1.fr/software/seaview
Texmaker v3.5.2	http://www.xm1math.net/texmaker/
Tracer v1.5	http://beast.bio.ed.ac.uk/

4 Methods

4.1 Study locations

Samples used in the present study originate from bats captured in West and Central Africa between 2006 and 2011 (figure 4.1). Except samples bought from hunters in Brazzaville, the capital of RC, all animals were captured in tropical rainforest areas. The author collected all samples from *Parc National d'Odzala-Kokoua* (PNOK) in RC in 2009, whereas the rest of the animals were captured and sampled by co-workers. Sampling procedure is described in section 4.2.

Estimates of chiropteran species diversity for each region vary from 18–41 (figure 4.1), however, these numbers might not reflect the true species diversity in the region since survey methods, sampling effort, and size of research areas differ markedly (J. Fahr, personal communication). Relevant information on the ecology of different sampling areas is given in the text. For detailed information on climate, vegetation, fauna, and known anthropogenic impact please refer to appendix A.2.

4.1.1 Choice of study sites

Samples from the northern region of the Republic of Congo, the *Parc National d'Odzala-Kokoua* (PNOK) and the logging concession south of Ouesso (*Industrie Forestière d'Ouesso*, IFO), should allow for testing the hypothesis stated by Walsh et al. (2005) regarding the wave-like spread of ZEBOV through Central Africa (Walsh et al. 2005): According to this theory the virus should spread from an outbreak region in Booue, Gabon, towards a north-eastern direction. This would imply a spread through the PNOK region, where latest filovirus-caused VHF outbreaks were reported in 2005, towards the IFO region, where no disease outbreaks have been reported to date. According to updated calculations the virus should hit the latter region by the time of sampling in 2010 (P. Walsh, personal communication). This scenario would result in distinct rates of virus detection and seroprevalence between animals from both regions. The latest VHF outbreak in the Taï region (PNT and PUTU) was reported in 1994 (Formenty et al. 1999), and there are no reports of filoviruses occurring in the region of the Trans-boundary Peace Park (GOLA) in Sierra Leone and Liberia. Samples from these areas therefore serve as 'control samples' and should display substanially lower seroprevalence.

Hunting, preparation, and consumption of bushmeat provide intensive contact between humans and wildlife. As mentioned above, bats are commonly hunted and sold on bushmeat markets. To

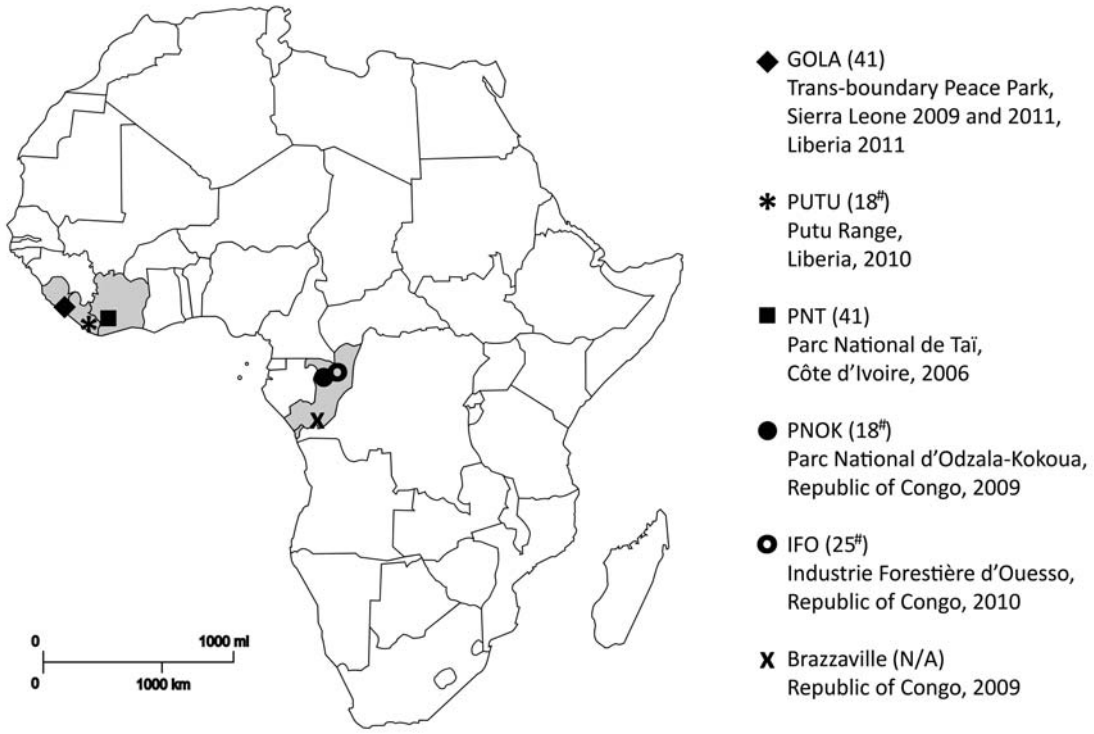


Figure 4.1: Map of Africa showing the origin of samples used in the present study. Numbers in parentheses indicate estimated number of chiropteran species diversity. #minimum number, incomplete surveys (J. Fahr, personal communication). N/A, not applicable

estimate the risk of pathogen transmission via bushmeat, samples from bats destined for selling on bushmeat markets in Brazzaville were investigated.

The *Parc National de Taï* was also chosen because it harbours a research site hosted by the Max Planck-Institute for Evolutionary Anthropology since 1977 (MPI EVAN; Leipzig, Germany). In 2001, following a number of unexplained death among chimpanzees, the Taï Chimpanzee Health Project was established by the MPI EVAN and the Robert Koch-Institute (RKI; Berlin, Germany), ensuring continuous monitoring of animals and sampling. Besides a tremendous number of non-invasive samples from healthy chimpanzees, and necropsysamples from deceased chimpanzees and any other wild animal found dead in the forest, samples from humans living in villages sourrounding the national park are also available. These samples were not analysed as part of this thesis, however, they offer the unique possibility for future follow-up studies.

4.2 Field Work

Part of the present work included a field trip to the Republic of Congo (RC) from July to October 2009 for capturing and sampling fruit bats and training local staff in *Ebolavirus* outbreak diagnostics.

During six weeks bats were captured on seven different locations within the *Parc National d'Odzala-Kokoua* (PNOK), located in the north-west part of RC. Bats might habour harmful pathogens and study sites included areas that have previously been heavily affected by Ebolavirus outbreaks, hence suitable safety measures were necessary: Briefly, personnel safety equipment included strong leather gloves for releasing animals from mist nets, FFP3 (*filtering face piece*) safety masks, face shields, Tyvec body-suits, and double layered gloves, the internal ones containing an inner disinfection layer (figure 4.2 B). Whenever possible disposable equipment was used (scalpels, forceps). Equipment and working place, including foil covered floor and table, was disinfected with bleach (chlorine) after each use. Carcasses were stored in formalin and burned with all rubbish when changing locations. Residuals and sharps were buried. Capture and handling of animals was only performed by trained personnel.

Figure 4.2: A: Mist net used for the capture of fruit bats; B: Performing necropsy on a captured bat; C: Taking a throat swab sample from an individual *Epomops franqueti*; D: Taking a blood sample from an individual *Megaloglossus woermanni*. Photos B and C by K. Nowak.

The focus of this field trip was to capture fruit bats since they are the main candidates for being filovirus reservoirs. In constrast to microbats, fruit bats fly rather high, hence mist nets were used to capture animals at heights up to 25 m (figure 4.2 A). To prevent birds from getting trapped nets were put up just before dusk and checked at least every hour. Nets stayed open until enough

animals had been trapped for sampling during the night and the next day (usually around 1 o'clock am). After having been collected from the nets, species and sex were recorded, and animals were put into cotton bags until further treatment. Up to 19 individuals were sampled during one night for throat swabs and blood (figure 4.2 C,D): Blood was taken with insulin syringes from the wing vein (*Vena cephalica*) and mixed with EDTA to prevent coagulation. Fecal samples, if available, were collected out of cotton bags and urine was collected directly in the cryovial or collected from the table surface with a syringe. Prior to release, bats were fed with fruit or honey. For safety reasons, necropsies were only performed during the day: Tissue samples were collected from those species known to carry relevant pathogens (mainly large fruit bats: *Hypsignathus monstrosus*, *Eidolon helvum*, *Epomops* sp. and *Myonycteris* sp.), and from individuals (mainly insectivorous species) taken for the reference collection of the University of Ulm. Therefore up to five individuals per night were provided with food (banana or papaya) and kept on a dry place overnight. The next day, throat swabs were taken before animals were anaesthetised with 0.6 ml per kg bodyweight of a 1:2 mixture of Rompun 2% (Xylazine 20 mg/ ml) and Ketamine (50 mg/ ml) by intramusclular injection into the pectoral musculature. For euthanasia animals were bled by cardiac puncture. Tissue samples were taken in the order spleen, liver, kidney, lung, salivary gland, small intestines. To minimize the risk of contamination the gastrointestinal tract had not been opened or damaged until all other organs were taken. Additional samples were obtained from animals destined for selling on bushmeat markets in Brazzaville. Animals were captured by local hunters close to Brazzaville and brought live to the National Laboratory in Brazzaville where they were euthanised and necropsies were performed, as described above for individuals captured in the field. All samples were stored in liquid nitrogen, transported on dry ice and transferred to -80°C at RKI for longtime storage. Alternatively, samples were stored in RNAlater and later transferred to -20°C for long time storage. In addition, tissue samples were preserved in 10% neutral buffered formalin for histopathologic analyses and stored at ambient temperature. Parasites (bat flies, ticks, mites) were also collected and preserved in 70% ethanol, but were not analysed in this study.
As part of a capacity enforcement programm local staff was trained in methods for *Ebolavirus* diagnostics during four weeks. This included extraction of viral RNA from various samples, sceening with an one-step reverse transcription real-time PCR, serological testing (antibody-capture ELISA and Western Blot), and interpretation of results (see sections 4.3.3 and 4.7 for further description of methods).

4.3 PCR-based pathogen detection

To screen for known pathogens *Polymerase Chain Reaction* (PCR) was used (Mullis et al. 1986). According to the pathogen of interest, different assay variations, namely conventional PCR, nested PCR, and quantitative real-time PCR (qPCR), were applied.

4.3.1 Extraction of nucleic acids

DNA and RNA were extracted from tissue samples using the NucleoSpin RNA II Kit. A lentil-size tissue piece was transferred to a bead tube containing lysis buffer, homogenised using a FastPrep cell disrupter for 20 sec, and further processed according to manufactures instructions. Nucleic acids were eluted in 60 µl RNase-free water and stored at -80°C. Fecal samples were extracted using the GeneMATRIX Stool DNA Purification Kit with addition of 5 µg carrier RNA per sample. Although designed for DNA extraction, the kit has been shown in our group to isolate RNA efficiently. Nucleic acids were eluted in 100 µl pre-heated RNase free water and stored at -80°C. Swab samples, urine, allantoic liquid, and cell culture supernatants were extracted using the QIAmp Viral RNA Mini Kit: Swab samples stored in RNAlater were vortexed for 1 min before use. Frozen swab samples were prepared by adding 500 µl PBS to the tube and vortexing for 1 min. PBS was added to urine samples if less than the final volume of 140 µl used for extraction was available. Samples were further processed according to manufactures instructions, nucleic acids were eluted in 60 µl RNase free water and stored at -80°C. No DNase digestion step was carried out during routine extractions.

4.3.2 cDNA synthesis

cDNA (*complementary DNA*) was synthesised by using the SuperScript II Reverse Transcriptase kit according to manufactures instructions by using random hexamer primers and adding the maximum amount of RNA.

4.3.3 PCR assays

Using various PCR assays, samples were screened for filoviruses, paramyxoviruses and hantaviruses. Primers and references are given in table 4.2, the PCR protocol and cycling conditions in table 4.1. PCR products from conventional PCR assays were analysed by agarose-gelelectrophoresis and results visualised under UV light.

Table 4.1: Protocol and cycling conditions for conventional PCRs

Mix	**[µl]**	**Cycling conditions**			
10x reaction buffer	2.5	95°C	300	sec	
dNTPs [2.5 mM]	2.0	95°C	15	sec	35x
$MgCl_2$ [50 mM]	2.0	T_a	35	sec	
forward primer [10 µM]	0.75	72°C	45	sec	
reverse primer [10 µM]	0.75	72°C	420	sec	
Platinum Taq Polymerase [5U/µl]	0.1				
template	5				
nuclease-free water	ad 25				

T_a=annealing temperature of respective primer

Table 4.2: Primers and probes for PCR screening assays. TM = TaqMan assay design

Pathogen	Name	Sequence	T_a [°C]	Reference
Filovirus (TM)	Filo A2.2	AAgCCTTTCCTAgCAACATgATggT	60	Panning et al. (2007)
	Filo A2.3	AAgCATTCCCTAgCAACATgATggT		
	Filo A2.4	AAgCATTTCCTAgCAATATgATggT		
	Filo B	ATgTggTgggTTATAATAATCACTgACATg		
	Filo B-Ra	gTgAggAgggCTATAAAAgTCACTgACATg		
	FAMEBOSu	6FAM-CCgAAATCATCACTIgTITggTgCCA-BHQ1		
	FAMEBOg	6FAM-CCAAAATCATCACTIgTgTggTgCCA-BHQ1		
	FAMMBG	6FAM-CCTATgCTTgCTgAATTgTggTgCCA-BHQ1		
Filovirus	Filo-A	ATCggAATTTTTCTTTCTCATT	37	Sanchez et al. (1999)
(single-round assay)	Filo-B	ATgTggTgggTTATAATAATCACTgACATg		
Filovirus	Greene-Filo-U12683-A	TATTCTCYCTACAAAAgCATTggg	50	Zhai et al. (2007)
(single-round assay)	Greene-Filo-L13294-A	gCTTCTgCgAgTgTTTggACATT		
	Greene-Filo-U12683-B	TATTTTCCATTCAAAAACACTggg		
	Greene-Filo-L13294-B	gCTTCACAAAgTgTTTgAACATT		
	Greene-Filo-U12683-C	TATTTTCAATCCAAAAgCACTggg		
	Greene-Filo-L13294-C	gCTTCgCAgAgggTTTggACATT		
	Greene-Filo-U12683-D	TATTCTCTgTTCAAAAACATTggg		
	Greene-Filo-L13294-D	gCCTCACATAAAgTTTggACATT		
Paramyxovirus	RES-MOR-HEN-F1	TCITTCTTTAgAACITTYggNCAYCC	50	Tong et al. (2008)
(seminested assay)	RES-MOR-HEN-F2	gCCATATTTTgTggAATAATHATHAAYgg		–modified–
–Paramyxovirinae–	RES-MOR-HEN-R	CTCATTTTgTAIgTCATYTTNgCRAA		
Paramyxovirus	PAR-F1	gAAggITATTgTCAIAARNTNTggAC	50	Tong et al. (2008)
(seminested assay)	PAR-F2	gTTgCTTCAATggTTCARggNgAYAA		–modified–
–Paramyxoviridae–	PAR-R	gCTgAAGTTACIggITCICCDATRTTNC		
Hantavirus	Han-L-F1	ATgTAYGTBAgTgCWgATgC	53	Klempa et al. (2006)
(nested assay)	Han-L-R1	AACCADTCWgTYCCRTCATC		
	Han-L-F2	TgCWgATgCHACIAARTggTC		
	Han-L-R2	gCRTCRTCWgARTgRTgDgCAA		

For quantification of virus load and to screen for paramyxovirus variations specific qPCRs were designed. The respective protocols are given in table 4.3, primers and probes in table 4.4.
Plasmid standards for qPCR-assays were prepared by growing the respective clones in 2 ml LB medium over night at 37°C. Plasmids were isolated using the Plasmid Mini Prep Kit according to manufactures instructions and copy numbers were determined. Before use, serial dilutions from 10^6 to 10^1 were prepared with lambda DNA to improve stability of plasmid DNA.

Table 4.3: Protocol and cycling conditions for quantitative real-time PCRs (qPCR)

Mix	**[µl]**	**Cycling conditions**		
10x reaction buffer	2.5	95°C	600 sec	
dN(U)TPs [2.5 mM]	2.0	95°C	15 sec	45x
$MgCl_2$ [50 mM]	2.0	60°C	35 sec	
forward primer [10 µM]	0.75			
reverse primer [10 µM]	0.75			
probe [10 µM]	0.25			
Platinum Taq Polymerase [5U/µl]	0.1			
template	3/5*			
nuclease-free water	*ad* 25			

*5 µl template when used for screening PCRs, 3 µl template for specific qPCRs

Table 4.4: Primers and probes for specific paramyxovirus qPCRs

Assay	Name	Sequence	T_a [°C]
Eidolon group A	Hen-Eid-A_F	AgTggACgATTgAggAg	60
	Hen-Eid-A_R	CCCCTCTCACTTgAYTCT	
	Hen-Eid-A_TM	6FAM-TgATggAAgggTAAACAgAgTC-BBQ	
Eidolon group B	Hen-Eid-B_F	TggCCYCCYCTAACCT	60
	Hen-Eid-B_R	AgCCCgCARAATgATYTC	
	Hen-Eid-B_TM	6FAM-CAATCATCAggggAAAgATTgACTTAT-BBQ	
Eidolon group C	Hen-Eid-C_F	TCAATTTggAgTgTTCATgCCTCT	60
	Hen-Eid-C_R	ggCgCCTTgAAACTTTACTCCT	
	Hen-Eid-C_TM	6FAM-ACCAgAAAAgAACgAATgggACYCAgT-BBQ	
Eidolon 214	Hen-Eid-214_F	CTTCCTCCCCATTgCTC	60
	Hen-Eid-214_R	TTgCgggATAgACAgAg	
	Hen-Eid-214_TM	6FAM-AgAATTggCAATCATTTTgTggAT-BBQ	
Eidolon 222	Hen-Eid-222_F	gAgATCAACATggAggTg	60
	Hen-Eid-222_R	gTAAACggAATCCCAATC	
	Hen-Eid-222_TM	6FAM-AAggCATTATCCCCATTgAAC-BBQ	
Eidolon 247	Hen-Eid-247_F	gCCCTCTTgTTCCCTTCCTg	60
	Hen-Eid-247_R	gTCATCATCCAgTgTCAAgggC	
	Hen-Eid-247_TM	6FAM-ATgAACggAgAggCATTgACAATAgAggA-BBQ	
Myonycteris group	Hen_Myo2_F	YggAAgAgCggTgAgAgT	60
	Hen_Myo2_R	CAATTggTgACAgTgCTTTATCTT	
	Hen_Myo2_TM	6FAM-AATgTTTTATgCCCCTTgAgTTggATAC-BBQ	

T_a: annealing temperature

4.3.4 DNA purification and cloning

PCR products were purified for sequencing using 2 µl ExoSap per 5 µl PCR product and incubation at 37°C for 15 min followed by 80°C for 15 min to inactivate enzyme. When multiple bands were visible in the agarose gel the band with the expected size was cut, purified using the Gel Extraction Spin Kit, and directly sequenced. If the DNA concentration was too low for direct sequencing or if double peaks were present after sequencing, a refresh-PCR (table 4.5) was performed on the original PCR product and the refresh-PCR product was TA cloned using the Topo TA Cloning Kit according to manufactures instructions. Colonies were analysed by colony PCR (using M13 primers provided with the Cloning Kit) and PCR products of insert containing clones were purified with ExoSap to be sequenced.

Table 4.5: Protocol for refresh-PCR

Mix	**[µl]**
nuclease-free water	9.38
10x reaction buffer	1.25
dNTPs [2.5 mM]	1.00
$MgCl_2$ [50mM]	0.75
Platinum Taq Polymerase [5U/µl]	0.13
Mix vol. per sample	10.00
purified PCR product	4.00
Incubation	70°C for 10 min

4.3.5 Sequencing

Sequence reactions were performed according to Sanger et al. (1977) on an ABI Prism 3100 Genetic analyser using the ABI Big Dye Termination Kit (table 4.6). PCR products were sequenced using the same primers as during amplification. Cloned sequences were generated with the M13 primers from the Topo TA Cloning Kit.

Table 4.6: Sequencing protocol

fragment size	**100-500 bp**	**500-1000 bp**	**Cycling conditions**		
Primer	0.5 µl	0.5 µl	96°C	2 min	
Big Dye	1.0 µl	2.0 µl	96°C	10 sec	25x
ABI Buffer	1.5 µl	1.0 µl	T_a	5 sec	
nuclease-free water	ad 10 µl	ad 10 µl	60°C	4 min	
DNA	see below	see below	T_a= annealing temp. of primers		
PCR products	100-200 bp	1-3 ng			
	200-500 bp	3-10 ng			
	500-1000 bp	10-20 ng			

4.4 Phylogenetic analyses of detected viruses

For many RNA viruses the genomic sequence of the *pol* gene (coding for the RNA-dependent DNA polymerase) shows a high degree of conservation and is therefore commonly used for phylogenetic analyses (Poch et al. 1990). Two different fragments were used for construction of phylogenetic trees of paramyxoviruses: The PAR fragment, which is conserved throughout the family *Paramyxoviridae* family, and the HEN-RES-MOR (HRM) fragment, which is conserved only among the genera *Henipa-*, *Respiro-*, and *Morbillivirus* in the *Paramyxovirinae* subfamily.
The software Geneious Pro was used to download and rename sequences from GenBank. Reference sequences for the PAR fragment were downloaded on 21st September 2011 and reduced to unique sequences using the FaBox program v1.4 (Villesen 2007). From those, separate datasets for all paramyxovirus genera were assembled and aligned on the amino acid level using the muscle algorithm as implemented in MEGA5 (Tamura et al. 2011). The best-fit model of nucleotide substitution was determined for all datasets using maximum likelihood-based Akaike information criterion scores as implemented in jModeltest 0.1.1 (Posada 2008). ML trees were generated with MEGA and for each genus the two sequences exhibiting the maximum patristic (= tree-based) distances were determined with Patristic version 1.0 (Fourment & Gibbs 2006). These sequences, whose selection guaranteed a reasonable representation of the whole diversity of each genus, were used to build up the final dataset. This additionally included all available paramyxovirus sequences from *Eidolon helvum* and those not assigned to an existing genus, thus comprising 62 sequences in total.
Reference sequences for the phylogenetic tree based on the HRM fragment were taken from (Drexler et al. 2012) and completed with all available bat paramyxovirus sequences available to date (17th August 2012), including sequences derived during this study. These added up to 214 sequences in total.
Sequences of the final datasets were aligned on protein level using the muscle algorithm (Edgar 2004) as implemented in the program SeaView version 4 (Gouy et al. 2010). To increase alignment quality conserved blocks were selected using the Gblocks server (Talavera & Castresana 2007). Conserved blocks were selected from protein alignments and reported on the corresponding nucleotide alignments. These were manually edited for final refinement and resulted in gapless 461 nt and 289 nt alignments for the PAR and the HRM fragments, respectively. The best-fit model of nucleotide substitution was determined as described above using jModeltest and resulted in selection of a general time reversible model (GTR) with gamma site heterogeneity (+G) and invariant sites (+I) for both fragments. This was used to inform phylogenetic analyses that were performed in both maximum likelihood (ML) and Bayesian frameworks. ML analyses were performed using the PhyML server (Guindon et al. 2010), and nearest-neighbor interchange (NNI) and subtree pruning and regrafting (SPR) algorithms were applied for tree search. Branch robustness was assessed by performing nonparametric bootstrapping (500 pseudo-replicates). Bayesian analyses were performed using the software package BEAST v1.7.0 (Drummond & Rambaut 2007). At least two independent runs of 25,000,000 generations were performed for each dataset under various tree priors and clock models. Trees and numerical values were taken every 1,000 genera-

tions. Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/) was used to check for convergence of individual runs, that independent runs converged on the same zones of parameter spaces, and that chain mixing was satisfactory (global effective sample size values above 200). After removal of a visually conservative 10% burn-in period, tree samples were gathered into a single file using LogCombiner (distributed with BEAST) and information was summarised onto the maximum clade credibility trees using TreeAnnotator (distributed with BEAST). Posterior probabilities (pp) were taken as a measure of branch robustness. Final trees were edited using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and the software Adobe Illustrator CS5.1 and Adobe Photoshop CS5.

Hantavirus sequences were downloaded on 2nd September 2011 and renamed using the software Geneious Pro. Sequences of all representative species listed on ICTV to date were used for phylogenetic analysis. Alignment and further processing of data was done as described above for paramyxoviruses. Phylogenetic analyses were performed in both ML and Bayesian frameworks under the assumption of a GTR+G+I nucleotide substitution model. The final tree was constructed using the software MEGA and edited using Adobe Illustrator and Photoshop.

4.4.1 Host switch reconstruction

To determine the locations and frequency of host switches made by paramyxoviruses all throughout the family evolutionary history the software Mesquite v2.75 (Maddison & Maddison 2011) was used. For this, a collection of Bayesian trees were generated, which were sampled during two independent runs of 25 mio generations and gathered into a single file after removal of a visually conservative 10% burn-in period. All parameters were resampled every 4,000 generations, resulting in a collection of 11,250 trees. The mammalian order to which the respective host species belonged was defined as a character of the viruses. Ancestral states of this character were then determined at all nodes (when possible) for all trees applying both, parsimonious and probabilistic approaches. The maximum number of mappings to sample for characters on each tree was set up to 50. Host switches were summarised as average number of state switches across all trees. The same analysis was run with randomly assigned hosts ('shuffled characters') to account for sampling bias.

4.5 Cell culture-based pathogen detection

For a selection of samples that were tested positive in PCR, virus isolation was attempted on various cell lines. A selection of blood and tissue samples was additionally used for blind virus propagation to allow for detection of formerly unknown viruses. Since it was not known if human-pathogenic viruses were present in the samples all experiments were done in a biosafety level 3 (BSL3) laboratory.

4.5.1 Cell lines and cultivation of cells

Blind virus propagation was done on VeroE6 and R05T cells. Vero cells are commonly used for virus propagation. They were derived from primate kidney cells and are interferon-type I-deficient, which makes them susceptible to many different viruses (Desmyter et al. 1968). R05T is a novel cell line established from fetal tissue of the bat species *Rousettus aegyptiacus* (Jordan et al. 2009). Isolation of paramyxoviruses was attempted on additional cell lines, namely rabbit and canine cocker spaniel kidney cells (RK-13 and MDCK II, respectively), and by infection of allantois liquid of embryonated chicken eggs. All cell lines (except R05T) have previously been used for successful isolation of paramyxoviruses (Smith et al. 2011; Wilkinson et al. 2012; Baker et al. 2012; Halpin et al. 2000; Jack et al. 2005; Lednicky et al. 2004).

All mammalian cell lines used here are adherent growing and were incubated in cell culture flasks at 37°C in a 5% CO_2 incubator. Growth medium (D-MEM) was supplemented with 1% L-glutamine and 5–10% FCS and cells were split every 3–4 days. Therefore, they were detached using a trypsin-EDTA solution (1:3) and resuspended in fresh medium in a proportion of 1:5. To characterise virus isolates two human cell lines (lung fibroblasts, MRC-5, and liver carcinoma cells, Hep2) and one insect cell line (mosquito larvae, C6/36) cells were used in addition. C6/36 cells were grown with L-15 medium supplemented with 1% L-glutamine and 5% FCS. Cells were incubated at 28°C without CO_2 and removed from flasks for splitting using a cell scraper.

4.5.2 Sample preparation

Blood samples from maximum eight individual bats per species (or less, if not enough individuals were available) from RCI were pooled by species in 46 pools and used to inoculate VeroE6 and R05T cells. Maximum 50 µl blood of each individual was used and diluted with PBS to a final volume of 2 ml which was used for inoculation of cells.
Tissue pieces (∼0.3 cm^3) from animals from RC were homogenised in 700 µl D-MEM without supplements using a Fast Prep cell disrupter. To avoid bacterial contamination of cell cultures tissue homogenates were filtered through a 0.45 µm filter and 30 µl of the filtrate was diluted 1:10 in D-MEM without supplements prior to inoculation of cells. In total, organ samples of 69 animals were mixed and processed together prior to inoculation. These correspond to those 'organ mix' samples tested for hantaviruses. Please refer to table A.1 for details on bat species. Additional organ samples (14 x liver, 14 x lung, 10 x spleen, 10 x small intestines) of *Eidolon helvum* were used separately to inoculate cells . Urine of paramyxovirus-positive animals was diluted 1:10 and 1:100 in PBS and used to infect VeroE6, R05T, RK-13, and MDCK II cells. The urine sample with the highest viral load was diluted 1:100 and used to infect embroynated chicken eggs.

4.5.3 Inoculation of cells

One day before infection, cells were counted in a Neubauer counting chamber. To get 70–80 % confluent cell layers the following day, 1.6 x 10^5 (VeroE6) or 8 x 10^4 (R05T) cells were seeded in

25 cm^2 cell culture flasks for inoculation with blood samples. For inoculation with tissue samples 2 x 10^5 or 1 x 10^5 cells for VeroE6 and R05T, respectively, were seeded on 24–well plates. Prior to inoculation medium was removed and cells were washed once with PBS and once with D-MEM without supplements. Cells were then inoculated with sample material processed as described above and incubated for 1 h at 37°C.
After initial incubation with blood samples, 3 ml of D-MEM (2 % FCS, 1 % L-glutamine, 1 % penicillin/ streptomycin) was added and incubated for another 24 h. The following day cells were washed twice with PBS and 5 ml new medium including all supplements was added. Cells inoculated with tissue sample supernatant were supplemented with 700 µl D-MEM (2 % FCS, 1 % L-glutamine). Medium on 24-well plates was replaced only if a toxic effect could be observed on the cells. All inoculated cell cultures were incubated for minimum seven days at 37°C with 5 % CO_2 and monitored daily microscopically for the presence of a cytopathic effect (CPE).
Ten days old embryonated chicken eggs were infected by injecting 100 µl urine-dilution into the allantois liquid. Eggs were incubated at 37°C and after seven days transferred to 4°C. The next day, allantois liquid was harvested and monitored for virus growth with qPCR.

4.5.4 Passaging of cells and supernatant

Supernatants of all samples were passaged minimum once. Medium was removed from cells and kept aside while cells were frozen at -80°C for 10 min to release viral particles potentially present in the cells. Cells and supernatant were mixed again and centrifuged 10 min at 8000 rpm to remove cell debris, supernatant was stored at -80°C, and 300 µl were used to inoculate new cells. When a change in cell growth behaviour was observed, cells and supernatant were passaged either until a clear CPE was visible or until cells appeared normal again with respect to uninfected control cells.

4.6 Virus isolation and characterisation

Vero cells infected with blood samples from Côte d'Ivoire (pool B30) were showing a cytopathic effect (CPE) in the second passage. When 80–100 % of the cells showed a clear CPE cells were harvested as described above and supernatant was used to infect a 175 cm^2 cell culture flask. Again, cells were harvested when a clear CPE was visible, transferred to a 50 ml centrifugation tube and centrifuged 10 min at 4600 rpm to remove cell debris. Supernatant was stored at -80°C for further analyses.

4.6.1 Ultracentrifugation of virus particles

To concentrate viral particles for following experiments a 175 cm^2 cell culture flask was infected and harvested as described above. After centrifugation, supernatant was ultracentrifuged through 5 ml of a 36% saccharose solution for 4 h at 28,000 rpm at 4°C in a Beckmann ultracentrifuge (rotor SW 32 Ti). Subsequently, supernatant was discarded and the pellet was allowed to dry for 10 min at room temperature before it was resuspended in 150 µl PBS over night at 4°C.

4.6.2 Visualisation of viral particles by electronmicroscopy

For visual localization of viral particles in cells, ultra-thin sections of infected cells were prepared. All electron microscopic experiments were done in collaboration with Dr. Andreas Kurth (Robert Koch-Institute, ZBS1).
Cells were grown in a 25 cm^2 cell culture flask and infected with supernatant of the pool B30. When a CPE became visible cells were fixed with GA (final concentration 2.5%) over night at 4°C. Photographic documentation of samples was performed on a FEI Tecnai G2 transmission electron microscope.

4.6.3 Virus titration

To determine the number of infectious virus particles and replication capacities of the virus, stocks were titrated on VeroE6 cells. Therefore 5.5 x 10^4 cells were seeded into 96-well plates. The next day cells were infected in six replicates with serial dilutions from 10^{-1} to 10^{-12}, and incubated for seven days before analysis. The dilution rate at which 50% of all cell cultures show a CPE is called the *tissue culture infectious dose 50* (TCID50). Virus titer was determined after (Reed & Muench 1938) according to the following equation:

$$\text{Titer/ml} = \frac{D^{(n/p+0.5)}}{D_0 DV}$$

D = dilution factor
n = number of CPE positive wells
p = number of replicates per dilution
D_0 = first dilution (not diluted = 1, 1:10 dilution = 0.1, etc.)
V = volume per well.

4.6.4 Virus growth kinetics

To determine the growth behaviour of viruses replication was monitored over time until the maximum virus titer was reached. Cells were infected with a multiplicity of infection (MOI) of 0.1. In contrast to routine infections the medium was completely removed after 1 h of incubation, cells were washed with PBS and supplied with fresh growth medium. Every 24 h supernatant was taken for RNA-extraction, cDNA synthesis and quantitative real-time PCR to determine copy numbers.

4.6.5 Determination of viral envelope

To determine the presence of a lipid envelope layer viral particles are treated with ether. If the virus is coated by a lipid envelope it should be protected and particles should remain infectious after treatment. Virus containing cell culture supernatant was diluted 1:10 in medium without supplements and mixed on ice for five minutes in a 1:1 mixture with diethylether. Ether was removed after centrifugation (10 min, 3000 rpm, 4°C) and supernatant was titrated as described

above. Examination was done microscopically after seven days. As a control virus containing supernatant was treated the same way but without ether.

4.6.6 Random amplification and sequencing

To gain sequence information of unknown viruses Particle-associated Nucleic Acid PCR (PAN-PCR; Stang et al. 2005) was applied to ultracentrifuged virus particles. Before extraction of nucleic acids a DNase treatment was performed on the resolved virus pellet to get rid of remaining cellular DNA particles. The principle of the PAN-PCR is shown on figure 4.3. Briefly, random primers were ligated to a fixed anchor-sequence (K-random primer) and hybridized with template DNA or RNA. Primers were extended either with T4 polymerase (DNA template) or with RNA-dependent DNA-polymerase (cDNA synthesis of RNA template). Using complementary anchor primers (K-primer) it was possible to amplify the DNA fragments which could then be used for TA-cloning and subsequent sequencing. Sequences were identified by using the blastn and blastx algorithms against GenBank entries (Altschul et al. 1990).

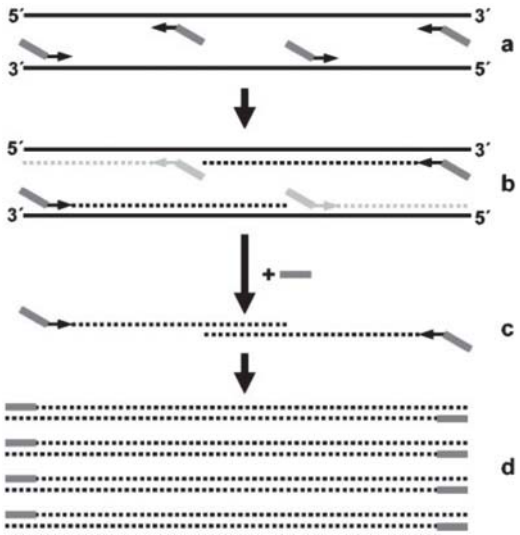


Figure 4.3: Mechanism of random PAN-PCR for RNA templates (adapted from Stang et al. 2005). (a) Hybridization of the randomized part of the K-random primer to template-RNA; (b) primer extension with RNA-dependent DNA-polymerase; (c and d) PCR with primer K: Hybridization and extension of strands generated in panel b (c) and PCR amplification with primer K (d). Continuous line, template-RNA; dotted line, newly synthesized (c)DNA-strand; small arrow, randomized part of K-random primer; shaded bar, constant part of K-random primer.

Resulting sequence contigs were assembled using the software SeqMan Pro of the DNASTAR Lasergene package and aligned with related virus species. To close sequence gaps 'out-Primers' were designed for PCR and sequencing. Please refer to figure 4.4 for a schematic description.

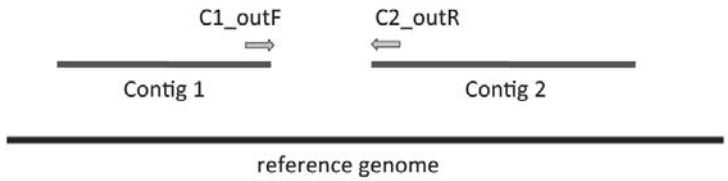


Figure 4.4: Schematic description of the sequencing strategy for the novel virus isolated from pool B30 using 'out-primers'.

Once partial sequence information were available, it was possible to test for genome orientation and whether the viral genome is single stranded (ss) or double stranded (ds). Since in this study the test was done on a RNA virus, it is described here accordingly. However, the method can easily be adapted to DNA viruses. For RNA genomes cDNA was synthesised using four different approaches: i) with random primers (R6), ii) with R6 without reverse transcriptase, iii) with a gen-specific forward primer and iv) with a gene-specific reverse primer. All four approaches were then used in a specific PCR reaction. For viruses with a RNA genome, approach ii) should not result in a PCR product. Approach iii) and iv) should only result in a PCR product when the genome has a negative or positive orientation, respectively, while for dsRNA genomes both approaches should yield a PCR product. Approach i) is the control reaction that should always be amplified correctly. Partial sequence information also allowed for designing a qPCR assay to screen cell cultures and to determine viral loads. Genome ends were amplified using the 5'RACE Kit for Rapid Amplification of cDNA ends according to manufactures instructions. As a modification, dsRNA was denaturated for 5 min at 94°C prior to cDNA synthesis. Due to the dsRNA genome the 5'RACE Kit could also be used to amplify 3' end of the segments by designing primers matching the 5' end of the complementary strand.

Table 4.7: Primers and probes used for sequencing of the virus from pool B30

	primer name	primer sequence	T_a [°C]
Segment 1	B30_C2_outF	TATgATTCAgCTCTggACgACgg	57
	B30_C4_outR*	CACACATCgCgATAATgAATgACTC	
	B30_C4_outF	ggTTgTgCAAggAgTggACgg	53
	B30_C1_outR	CTAgCCACCTTCCCTCTTCATC	
	B30_C1_outF	gAAATgTgAAggAAACTgTTgAgg	50
	B30_C3_outR	ATTgTgggTAAgTCATTTCATAAg	
	B30_C1_outF2	ggATggCggTAgATTggTATTATg	50
	B30_C1-3_outR	gTATCACACgCggTCTTCTC	
	B30_C3_outF	gAgCgACTTCTgTTATTTACTTCC	50
	B30_C5_outR	gACAggACgAgCAgAAAACTTgAg	
	B30_C2_outF_rev*	CCgTCgTCCAgAgCTgAATCATA	56
	B30_C2_outR*	CCgTCgTCCAgAgCTgAATCATA	57
Segment 2	B30_C8_outF	AATggATTAgggACCgTTgTTgAC	54
	B30_C10_outR	ggTCTCgCATTTCTCATCggTTCA	
	B30_C8_outF2	AATTTgAgAACggggAgAAgAAgT	52
	B30_C8-10_outR*	gggACAgCTCCATCAATACAgAC	
	B30_C8-10_outF	gAATggggAgCgTTgTTg	50
	B30_C10_outR2	CgTCTCggATCTggCTggTAT	
	B30_C10_outF	gTAgTACgTgCAggggAggTggAg	55
	B30_C6_outR	ggCgTAAAAggAgAAAAgTggATg	
	B30_c8-10_outF2	gTTgCgAgAgAATCCggACg	55
	B30_C10_outR3	TCCCTCCATACCTTgCCACC	
	B30_c10_outF2	TAgTTgACCCCgTgAgACgC	55
	B30_c6_outR2	gTAACCCCATCAACggTgCC	
	B30_C8_outR*	AAACCTATCAgCTgAATCCCCTCC	57
	B30_C8_outF_rev*	gTCAACAACggTCCCTAATCCATT	55
Segment 3	B30_s3c1_outF	CggggATCggAATgAgggAA	55
	B30_s3c2_outR	TgAgACgATgAgAggggCTg	
	B30_s3c2_outF	ACAgCCCCTCTCATCgTCTC	55
	B30_s3c3_outR	AggCgCAggCgAAATTATCC	
TaqMan	B30_F1	TggAgAgAgACATCAggTAgggAg	60
	B30_R1	gTAcccAgTTTTcTTATAcgccg	
	B30_TM	6FAM-TggTTgggACggAAgggCAg-BBQ	

*gene specific primers used for 5'RACE

4.6.7 Whole genome sequencing

RNA of the virus isolate was also prepared to be used for whole genome sequencing using the Roche 454 sequencer. This yielded in 222,953 reads with an average length of 372 bases for a total of 83 million bases. Data processing was done by Wojtek Dabrowski (Robert Koch-Institute, ZV4). Human DNA background was removed through mapping against the human genome 19 sequence (available from USCS Genome Bioinformatics 2012) using the software Newbler v2.6 (distributed by Roche) and discarding all matching reads. The remaining 91,454 reads were assembled together with the previously obtained Sanger sequences using both mira (Chevreux et al. 2000) and Newbler. Contigs from both assemblies were compared to a database of all reovirus sequences present in GenBank (blastn algorithm; Altschul et al. 1990, word size: 7, e-value: 100) using Geneious Pro. Nine contigs from the Newbler assembly, 23 contigs from the mira assembly, and available Sanger sequences were reassembled together using Geneious Pro

and resulted in nine final contigs. For error correction, all reads were then mapped against these contigs and discrepancies were resolved manually.

4.6.8 Sequence analyses

Nucleotide sequences were analysed with SeqMan Pro v10.0.1 and the software Geneious Pro. All contigs and remaining single sequences were compared and identified with GenBank entries using the blastn and the tblastx algorithm (Altschul et al. 1990) as implemented in Geneious Pro. Possible open reading frames (ORFs) were identified using the ORF finder from NCBI (NCBI) and putative protein functions were assigned by comparison with UniProt entries (The Uniprot Consortium 2012).

4.7 Serology

Filoviruses can only be handled in high security labs (BSL4) so antigen preparation was performed by Dr. Verena Krähling, Insitut für Virologie, Philipps-Universität Marburg. VeroE6 cells were infected with either EBOV-Zaire strain Mayinga, (accession number NC_002549) or MARV strain Musoke (accession number NC_001608.3) and cell lysate or purified virus antigen were prepared. For preparation of cell lysate cells were scraped six days post infection, centrifuged (10 min 1,000 rpm) and resuspended in PBS. Then, sodium dodecyl sulfate (SDS) was added at a final concentration of 1%. By boiling for 10 min at 99°C samples were inactivated and could be removed from the BSL4 facility. Antigen was prepared by ultracentrifugation of supernatants of MARV infected Vero cells through a 20% sucrose cushion (2 h, 4°C at 76,300 x g). The virus pellet was resuspended in PBS and inactivated by addition of SDS (final conc. 1%) and boiling for 10 min at 99°C before it was removed from the BSL4 facility.

4.7.1 Sample preparation

Whole blood samples were stored differently according to field conditions: Samples collected in the Taï National Park in 2006 and in the Republic of Congo 2009 (PNOK and Brazzaville) were frozen in liquid nitrogen. For use in serological tests samples were thawn completely, diluted first 1:10 in PBS, and subsequently again 1:10 in PBS including 0.1% Tween 20 (PBS-T) containing 1% (w/v) skimmed milk powder (PBS-T/M). Samples collected in the Gola National Park (GOLA) in Sierra Leone and in RC in 2010 (IFO) were stored as dried blood spots on filter paper. Blood spots were solved over night at 4°C in 500 µl PBS and subsequently diluted 1:10 with PBS-T/M.

4.7.2 Antibody capture ELISA

Cell lysate from infected cells was diluted 1:2000 in PBS and allowed to adsorb to Microtiter plates over night at 4°C (50 µl/ well). The next day, plates were washed 3 x 10 min with PBS-T (300 µl/ well). Skimmed milk powder was diluted in PBS-T to 5% and used for blocking during 1 h. Plates were washed again and incubated for 1 h with prepared serum samples. After an additional washing step a peroxidase-labelled (POD) goat anti-bat antibody was diluted 1:1000

in PBS-T/M and incubated for 1 h. The last washing step was performed 2 x 10 min with PBS-T and subsequently 2 x 10 min with PBS before SureBlue TMB Microwell Substrate was added. After 10 min SureBlue TMB Stop Solution was added to stop the reaction and plate was read at the wavelength of 450 nm (reference 650 nm). Anti-filovirus goat serum served as positive control (diluted 1:10,000 in PBS-T, 1% milk) and was detected with a POD-labelled donkey anti-goat antibody (diluted 1:1000 in PBS-T, 1% milk). All steps were carried out at room temperature.

4.7.3 Evaluation of ELISA

For normalization of optical density (OD) values from different plates all values were related to the goat serum which was used as positive control on each plate. Data was further processed using the software R (R Development Core Team 2012). Samples were considered reactive when their respective OD value was found to be an outlier according to the Smirnov-Grubbs rejection test included in the package outliers (Komsta 2011). Briefly, the test determines the ratio between the highest value of a given dataset (the putative outlier) and the mean of the dataset and divides it by the standard deviation. If this ratio is above a certain threshold the value represents an outlier and hence the sample is considered reactive. The respective OD value is discharged, and the second highest OD value is taken and tested accordingly. This is repeated until the p-value falls below the level of statistical significance. To be conservative, the p-value was set to $p < 0.01$. A two-sided Fisher's exact test (p-value 0.05) was performed to test whether there is any influence of age or gender on the outcome of the results.

4.7.4 Immunoblot (Western blot)

Samples reactive in ELISA were analysed by immunoblot for confirmation. SDS gels were prepared according to table 4.8. For preparative gels 10 µl virus antigen was diluted 1:3 in PBS or 30 µl of infected cell lysate was used and mixed 1:2 with Laemmli buffer, boiled for 5 min and cooled down on ice. As size marker 3 µl Fermentas Page Ruler Protein Ladder Plus was used. Gels were run at 20 mA/ gel for 1.5 h.

Table 4.8: Protocol for preparation of SDS gels

	12% separation gel (10 ml)	**5% stacking gel (3 ml)**
Aqua dest	3.3 ml	2.1 ml
30% NF-Acrylamid/Bis-solution (29:1)	4.0 ml	500 µl
Tris (1.5 M, pH 8.8)	2.5 ml	-
Tris (1 M, pH 6.8)	-	380 µl
SDS (20%)	50 µl	30 µl
APS (10%)	100 µl	30 µl
TEMED	10 µl	3 µl

After separation, proteins were semi-dry blotted onto a nitrocellulose membrane for 1.5 h at 44 mA/ gel, subsequently washed 3 x 10 min with PBS-T, and directly blocked over night at 4°C with 10% milk in PBS-T. The next day, blots were washed, dried and stored at -20°C until further

use. Before the first use each membrane was tested with a positive control (positive goat serum 1:10,000; donkey anti-goat POD 1:100,000) to confirm protein transfer onto the membrane. For each sample, a 0.5 cm slice was cut from the membrane and used for testing. Serum samples and antibody samples were diluted in PBS-T containing 1% milk and incubated for 1 h. After each incubation step blots were washed 3 x 10 min with PBS-T. The last washing step was 2 x 10 min with PBS-T, then 2 x 10 min with PBS. SuperSignal West Dura Chemiluminescent Substrate was used for detection on films. Samples were considered positive if they reacted with at least one filovirus protein.

5 Results

5.1 Sampling

Overall, more than 3600 samples were available from ∼1300 individuals belonging to 30 different genera. Of these, 203 bats were captured and sampled by the author during a field trip to the *Parc National d'Odzala-Kokoua* (PNOK) in the Republic of Congo (RC) in August and September 2009; an overview about the different species captured is given in figure 5.1. Of the captured individuals, 152 were released after blood, throat swabs and, if possible, fecal and urine samples were taken. The remaining 51 animals (mainly fruit bats) were euthanised to obtain organ samples.

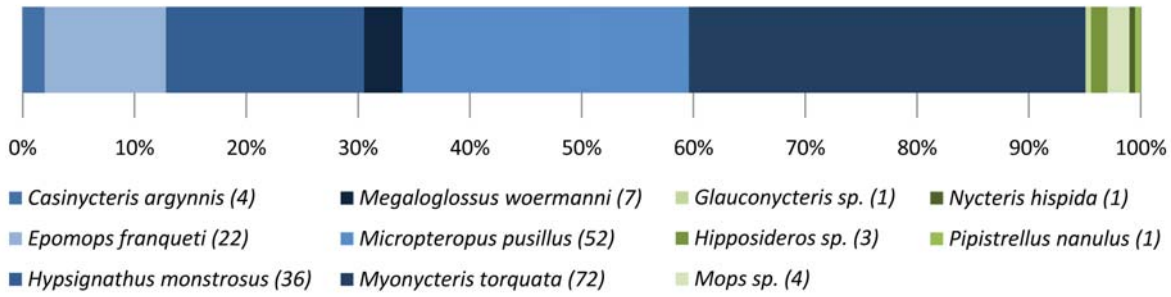


Figure 5.1: Distribution of bat species captured in the *Parc National d'Odzala* (Republic of Congo) in August and September 2009. Frugivorous species are represented in blue, insectivorous species in green. Numbers in parenthesis are captured individuals per species

Animals were captured opportunistically, with the focus on fruit bats (= Pteropodidae). Accordingly, the majority (193; 96%) of the animals captured in PNOK were pteropodid bats and only nine (5%) were insect feeding animals. Less than half (48%) of the animals were male while 52% were female. Another 42 animals were bought from local hunters in Brazzaville, the capital of RC, in October 2009. Of these, 29 (69%) were female and only 13 (31%) were males. All individuals were *Eidolon helvum* and destined for selling on bushmeat markets in town.

5.2 PCR Screening of bat samples

Bat tissue and blood samples were screened with generic PCR systems for selected virus families with known zoonotic potential.

5.2.1 Hantavirus in bat from Sierra Leone

To investigate the true host range of hantaviruses, a total of 356 tissue and blood samples from 247 bats representing 26 genera were tested for the presence of hantavirus RNA by genus-specific RT-PCR. Samples originated from Sierra Leone, Liberia, Côte d'Ivoire, Senegal, and the Republic of Congo and were collected during 2009 and early 2011. Details about tested samples and their origin are given in appendix A.3. One sample yielded a product of the expected size and was subjected to cloning and sequencing. The positive sample (MGB/1209) was obtained from one of 18 investigated slit-faced bats (Nycteridae, figure 5.2). The animal was a male adult trapped at the Magboi River within Gola National Park, Sierra Leone (7°50.194'N, 10°38.626'W), and the identification as *Nycteris hispida* has been verified with the voucher specimen (RCJF529). Consequently, the novel virus was named *Magboi virus* (MGBV). Histologic examination of organs of the animal (performed by K. Nowak) revealed no obvious pathologic findings.

Figure 5.2: *Nycteris hispida* (RCJF529). Photo by K. Nowak.

The obtained 414 nt sequence covers a genomic region, which was found to correspond to nt position 2,918-–3,332 in the large segment open reading frame of prototypic *Hantaan virus*. Bioinformatic analysis on the amino acid level showed highest degrees of identity to shrew- and mole-associated hantaviruses (*Thottapalayam virus* 73.0%, *Altai virus* 69.7%, *Nova virus* and *Imjin virus* 69.3%), as well as to a newly published bat virus (*Mouyassué virus* 64.1%). On the basis of tree topology of a maximum-likelihood phylogenetic tree, the sequence does not cluster with rodent-associated hantaviruses but groups with those found in shrews and moles (figure 5.3). Sequencing of the full genome of MGBV was impaired by low nucleotide identity impeding primer design and a lack of material suitable for whole genome sequencing or isolation attempts.

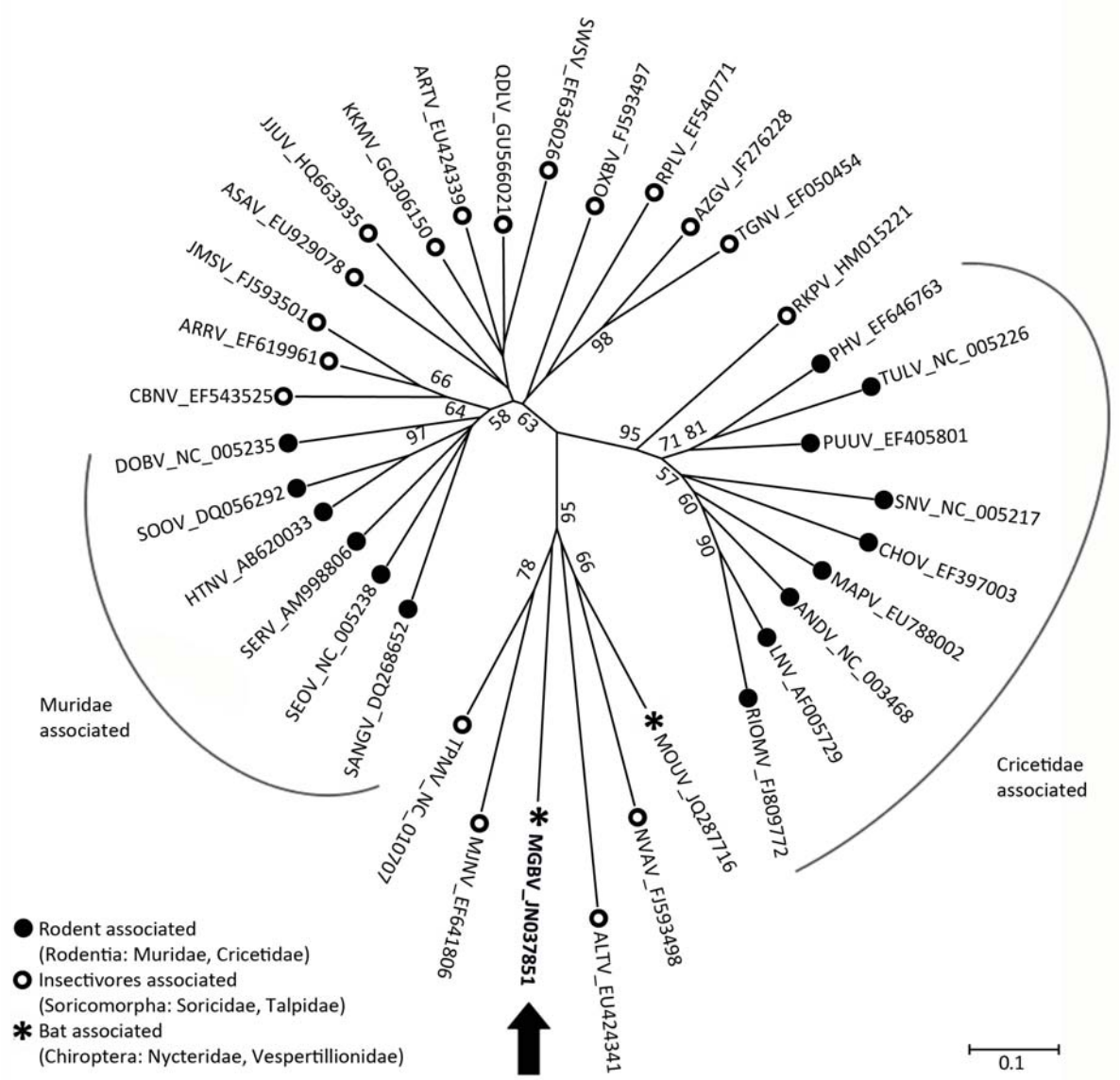


Figure 5.3: Maximum-likelihood phylogenetic tree of hantaviruses based on a 332 nt gapless alignment of large segment sequence. The tree was computed with the MEGA5 based on the general time reversible model with gamma distributed rate heterogeneity and invariant sites (GTR+G+I). Values at tree branches are bootstrap support values for 500 replicates (given in % when above 55). The scale bar indicates an evolutionary distance of 0.1 substitutions per position in the sequence. Chiroptera associated viruses: MGBV (*Magboi virus*), MOUV (*Mouyassué virus*); Rodentia associated viruses: ANDV (*Andes virus*), CHOV (*Choclo virus*), DOBV (*Dobrava-Belgrade virus*), HTNV (*Hantaan virus*), LNV (*Laguna Negra virus*), MAPV (*Maporal virus*), PHV (*Prospect Hill virus*), PUUV (*Puumala virus*), RIOMV (*Rio Mamore virus*), SANGV (*Sangassou virus*), SEOV (*Seoul virus*), SERV (*Serang virus*), SNV (*Sin Nombre virus*), SOOV (*Soochon virus*), TULV (*Tula virus*); Soricomorpha associated viruses: ALTV (*Altai virus*), ARTV (*Artybash virus*), ASAV (*Asama virus*), ARRV (*Ash River virus*), AZGV (*Azagny virus*), RPLV (*Camp Ripley virus*), CBNV (*Cao Bang virus*), MJNV (*Imjin virus*), JJUV (*Jeju virus*), JMSV (*Jemes Springs virus*), KKMV (*Kenkeme virus*), NVAV (*Nova virus*), OXBV (*Oxbow virus*), QDLV (*Qiandao Lake virus*), RKPV (*Rockport virus*), SWSV (*Seewis virus*), TGNV (*Tanganya virus*), TPMV (*Thottapalayam virus*).

5.2.2 Paramyxoviruses in African bats

To investigate pathogen transmission risk by the use of bats as bushmeat, a total of 902 samples from 299 individuals belonging to three genera were tested for infection with paramyxoviruses: 51 were the straw-coloured fruit bat *Eidolon helvum*, 196 the little collared fruit bat *Myonycteris* sp., and 52 individuals belong to *Hipposideros* sp. (figure 5.4). For details please refer to table 5.1. *Eidolon* samples were chosen for analysis mainly because of their close relationship to Australoasian pteropodid bats, which are known to harbour human pathogenic PMVs. *Eidolon helvum* are migratory animals and are most frequently hunted as bushmeat, thus posing a risk for transmission to humans. *Myonycteris* species, in contrast, are less frequently consumed by humans and do not show long-distance migratory behaviour. Combining knowledge on viruses harbored by these animals with information on phylogeography of west and central African *Myonycteris* populations could give insight into the virus dynamics of PMVs in African bats. *Hipposideros* represents the most speciose bat genus and was chosen as representative of insectivorous bats because of its close relationship to Pteropodidae, as compared to other microbats.

Of all samples, 33 (3.7%) were tested positive and 26 (2.91%) yielded a sequence. Samples originated from different field sites in West and Central Africa for which the proportion of individuals sampled from each species varied considerably (figure 5.4). This variation is likely due to sampling bias and does not necessarily reflect differences in species densities.

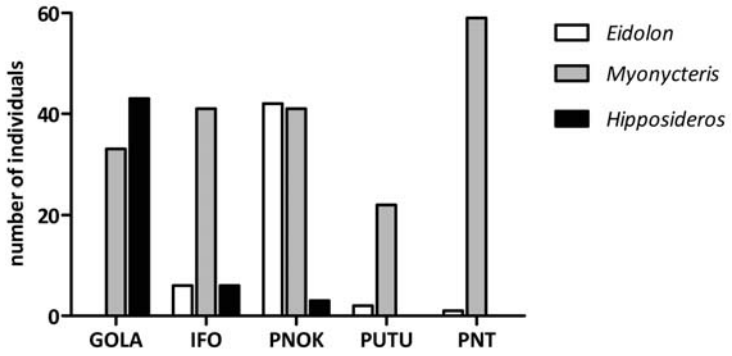


Figure 5.4: Origin of samples tested for paramyxoviruses. PNT, *Parc National de Taï*, Côte d'Ivoire; PNOK, *Parc National d'Odzala-Kokoua* (including samples from Brazzaville), Republic of Congo; IFO, *Industrie Forestière d'Ouesso*, Republic of Congo; GOLA, *Trans-boundary Peace Park*, Sierra Leone and Liberia; PUTU, *Putu Range*, Liberia.

The highest detection rate was observed in spleen (16.4%), followed by urine and kidney (4%), and liver (3.1%). Positive tested samples from *E. helvum* include twelve spleen, three kidney, one liver, and two urine samples. These originate from 14 individuals that were all bought from hunters in Brazzaville, RC (4°22'40"S, 15°06'27"E), and accounted for 33% of all animals (n = 42) destined for selling on bushmeat markets. Seven individuals (3.6%) of *My. torquata* from Central Africa (PNOK and IFO) tested positive. The samples include six spleen, two liver and one sample each from small intestines, kidney, lung, feces, and urine. Positive samples from *Hipposideros* sp.

Table 5.1: Samples tested for paramyxoviruses with PCR. Number of tested samples/ number of samples tested positive; #saliva from throat swabs, one salivary gland each fruit bat species tested negative; *no sequence available, one sample only tested positive in qPCR. PNT, *Parc National de Taï*, Côte d'Ivoire; PNOK, *Parc National d'Odzala-Kokoua* (including samples from Brazzaville), Republic of Congo; IFO, *Industrie Forestière d'Ouesso*, Republic of Congo; GOLA, *Trans-boundary Peace Park*, Sierra Leone and Liberia; PUTU, *Putu Range*, Liberia. amn. fluid, amniotic fluid; oral muc., oral mucosa, small int., small intestines; phar.tissue, pharyngeal tissue; plac., placenta.

Sample Site/ Genus	**amn. fluid**	**blood**	**feces**	**heart**	**small int.**	**kidney**	**liver**	**lung**	**milk**	**phar. tissue**	**plac.**	**spleen**	**saliva#**	**urine**	**total**
GOLA			**26**		**1**	**1**	**9**				**1**	**14/1**	**34**	**13**	**99/1**
Hipposideros			26				8				1	13/1	1	10	**59/1**
Myonycteris					1	1	1					1	33	3	**40**
IFO	**1**		**34**		**28/1**	**31/1**	**31/1**	**5/1**	**1**			**33/4***	**6**	**16**	**186/8***
Eidolon			5		2	3	3	3				3	5		**24**
Hipposideros			6						1			2		1	**10**
Myonycteris	1		23		26/1	28/1	28/1	2/1				28/4*	1	15	**152/8***
PNOK		**34**	**55/2**		**68**	**69/3**	**88/3**	**44**				**68/14***	**54**	**46/3***	**526/24***
Eidolon		33	31		42	42/3	41/1	42				42/12*	39	21/2*	**333/18***
Hipposideros			2/1		1							1		1	**5/1**
Myonycteris		1	22/1		25	27	47/1	2				25/2*	15	24/1	**188/5***
PUTU							**1**						**23**		**24**
Eidolon													2		**2**
Myonycteris							1						21		**22**
PNT		**59**		**1**			**1**	**1**		**1**		**1**	**1**		**65**
Eidolon													1		**1**
Myonycteris		59		1			1	1		1		1			**64**
Total	**1**	**93**	**115/2**	**1**	**97/1**	**101/4**	**130/4**	**50/1**	**1**	**1**	**1**	**116/19***	**118**	**75/3***	**902/33***

stem from one spleen and one fecal sample from two distinct individuals (7.7%), one from West and one from Central Africa (GOLA and PNOK), respectively.

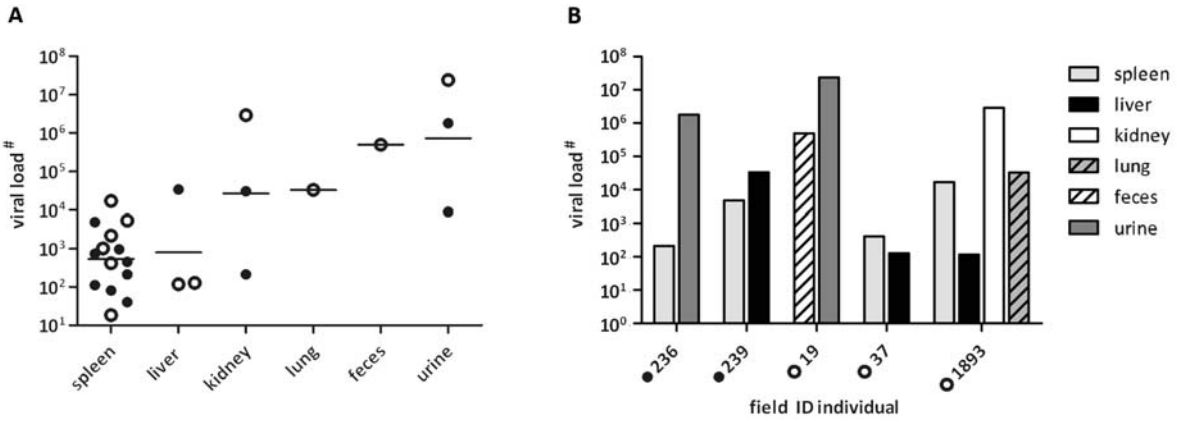


Figure 5.5: Paramyxovirus quantities in all positive tested samples. #Viral loads are given per piece of organ for feces (∼0.3 cm^2) or per ml urine. A: Black circles represent *Eidolon helvum* samples, open circles represent *Myonycteris torquata* samples; line indicates geometric mean of all samples. B: Viral loads in single individuals with multiple infected organs.

Viral load differed widely depending on individual and material (figure 5.5). Quantities were determined by real-time PCRs (qPCR) which were also used to re-screen spleen, kidney and urine samples of *E. helvum*. Two spleen samples (field IDs 220 and 221) and one urine sample (field ID 236) displayed additional positive results in qPCR, but no sequences were derived for these samples, due to limited material. Detection rate was the highest in spleen. However, viral load was rather low in spleen, but high in urine (table 5.5). One individual of the straw-coloured fruit bat (field ID 236) *Eidolon helvum* was at least double infected: For the HRM fragment, two distinct sequences were retrieved from the spleen sample; one of these was identical with the urine-derived sequence. The urine sample, in contrast, yielded two distinct sequences in the PP fragment. Only one individual (*My. torquata* field ID 1893) yielded identical sequences in all organs tested (spleen, liver, kidney, lung, intestines), but was tested negative for PMV in urine.

Phylogenetic analyses of detected paramyxovirus sequences To further analyse the genetic relationship between the novel bat-derived paramyxovirus (PMV) sequences and known PMV sequences, phylogenetic trees were generated based on different regions from the L-gene (*pol*) (see section 4.4). One region (PAR fragment) is conserved throughout the PMV genera (figure 5.7) while the second one (HRM fragment) is only well conserved within the genera *Respiro-*, *Morbilli-* and *Henipavirus* (figure 5.6). Tree topologies are in concordance with the general agreement of PMV division and did not reveal relevant changes when different tree priors and speciation models were applied in Bayesian analyses (data not shown). Final trees were constructed under the assumption of a relaxed, uncorrelated lognormal clock and the Yule process speciation model. Novel Eidolon paramyxovirus (EPMV) sequences group with other EPMVs originating from Ghana, West Africa, within the subfamily *Paramyxovirinae* and form a sister-clade to the genus *Henipavirus* in both trees (PAR- and HRM-based).

Table 5.2: Origin, quantities and accession numbers of samples tested positive for paramyxoviruses (HRM fragment). n.a., not available; M, male; F, female; ad, adult; juv, juvenile; 'no sequence available, sample only tested positive in qPCR; *no measurable quantity probably due to too low copy numbers; $^{+}$Eidolon group-A (qPCR); $^{\$}$Eidolon group-B (qPCR); $^{\&}$Eidolon group-C (qPCR); samples printed in bold were used for isolation attempts; $^{\%}$ copy number per piece of organ or feces (∼0.3 cm^2) or per ml urine.

Species	field ID	sex	age	sample	site	copy number$^{\%}$	accession number
E. helvum	210^{+}	M	juv	spleen	Brazzaville	1.11E+02	HE647821
	214	F	ad	**spleen**	Brazzaville	neg	HE647822
	215$^{\$}$	M	n.a.	**spleen**	Brazzaville	9.55E+02	HE647823
	216$^{\$}$	M	n.a.	**spleen**	Brazzaville	7.30E+02	HE647824
	220$^{\&}$	F	ad	spleen	Brazzaville	8.46E+01	n.a.'
	221$^{\&}$	F	n.a.	spleen	Brazzaville	8.07E+01	n.a.'
	222	F	n.a.	**kidney**	Brazzaville	2.11E+02	HE647825
	226$^{\$}$	M	n.a.	spleen	Brazzaville	neg*	HE647826
	236^{+}	F	juv	spleen	Brazzaville	2.13E+02	HE801055 HE801056
	236^{+}	–	–	**urine**	Brazzaville	1.80E+06	HE647827
	237$^{\&}$	M	juv	spleen	Brazzaville	neg*	HE647828
	237$^{\&}$	–	–	urine	Brazzaville	8.89E+03	n.a.'
	239^{+}	F	juv	**kidney**	Brazzaville	neg*	HE647829
	239^{+}	–	–	**liver**	Brazzaville	3.35E+04	HE647830
	239^{+}	–	–	**spleen**	Brazzaville	4.83E+03	HE647831
	240^{+}	M	juv	spleen	Brazzaville	4.49E+02	HE647832
	241$^{\&}$	F	juv	spleen	Brazzaville	neg*	HE647833
	247	M	juv	kidney	Brazzaville	3.02E+04	HE647834
M. torquata	19	F	n.a.	fc	PNOK	4.96E+05	HF679389
	19	–	–	urine	PNOK	2.38E+07	HF679390
	37	F	n.a.	liver	PNOK	1.28E+02	HF679391
	37	–	–	spleen	PNOK	4.16E+02	n.a.'
	51	M	n.a.	spleen	PNOK	5.30E+03	HF679388
	1893	M	juv	int	IFO	neg*	HF679397
	1893	–	–	kidney	IFO	2.90E+06	HF679393
	1893	–	–	**liver**	IFO	1.17E+02	HF679394
	1893	–	–	**lung**	IFO	3.28E+04	HF679395
	1893	–	–	spleen	IFO	1.69E+04	HF679396
	1903	M	ad	spleen	IFO	2.16E+03	HF679399
	2035	M	ad	spleen	IFO	1.01E+03	n.a.'
	2072	F	ad	spleen	IFO	1.85E+01	HF679398
***Hipposideros* sp.**	21	F	n.a.	fc	PNOK	n.a.	HF679402
	1150	M	ad	spleen	GOLA	n.a.	HF679403

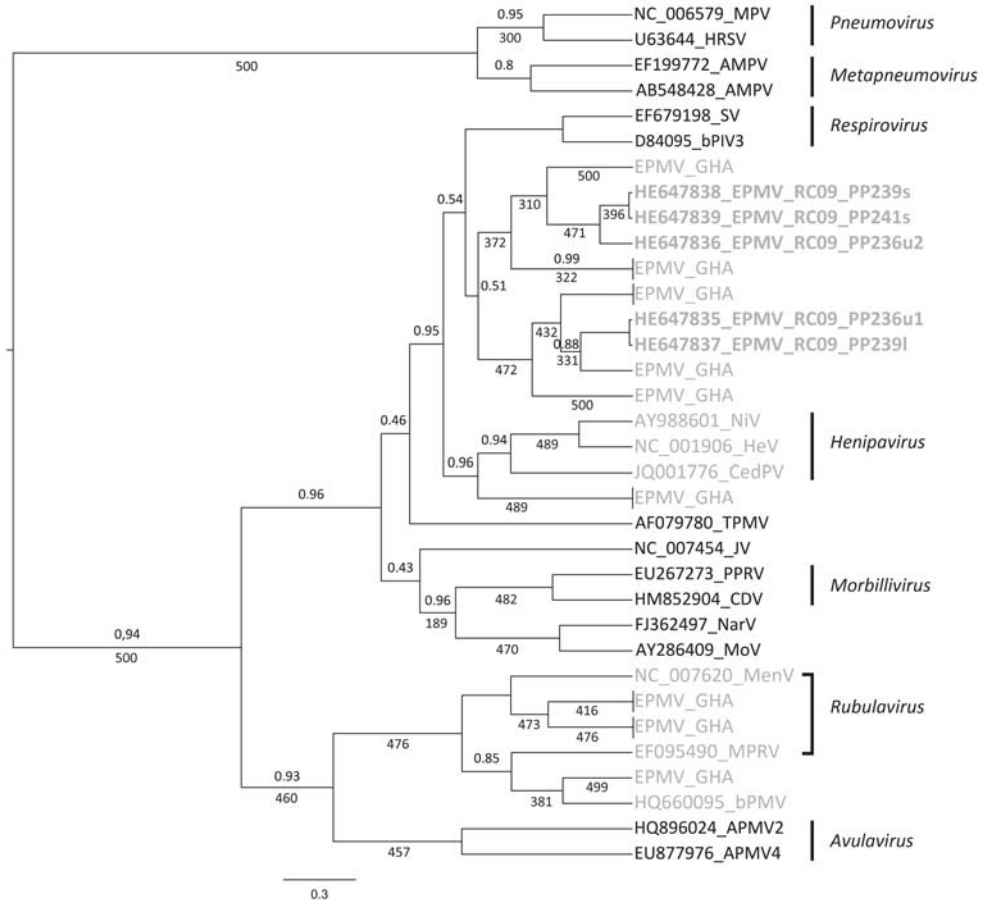
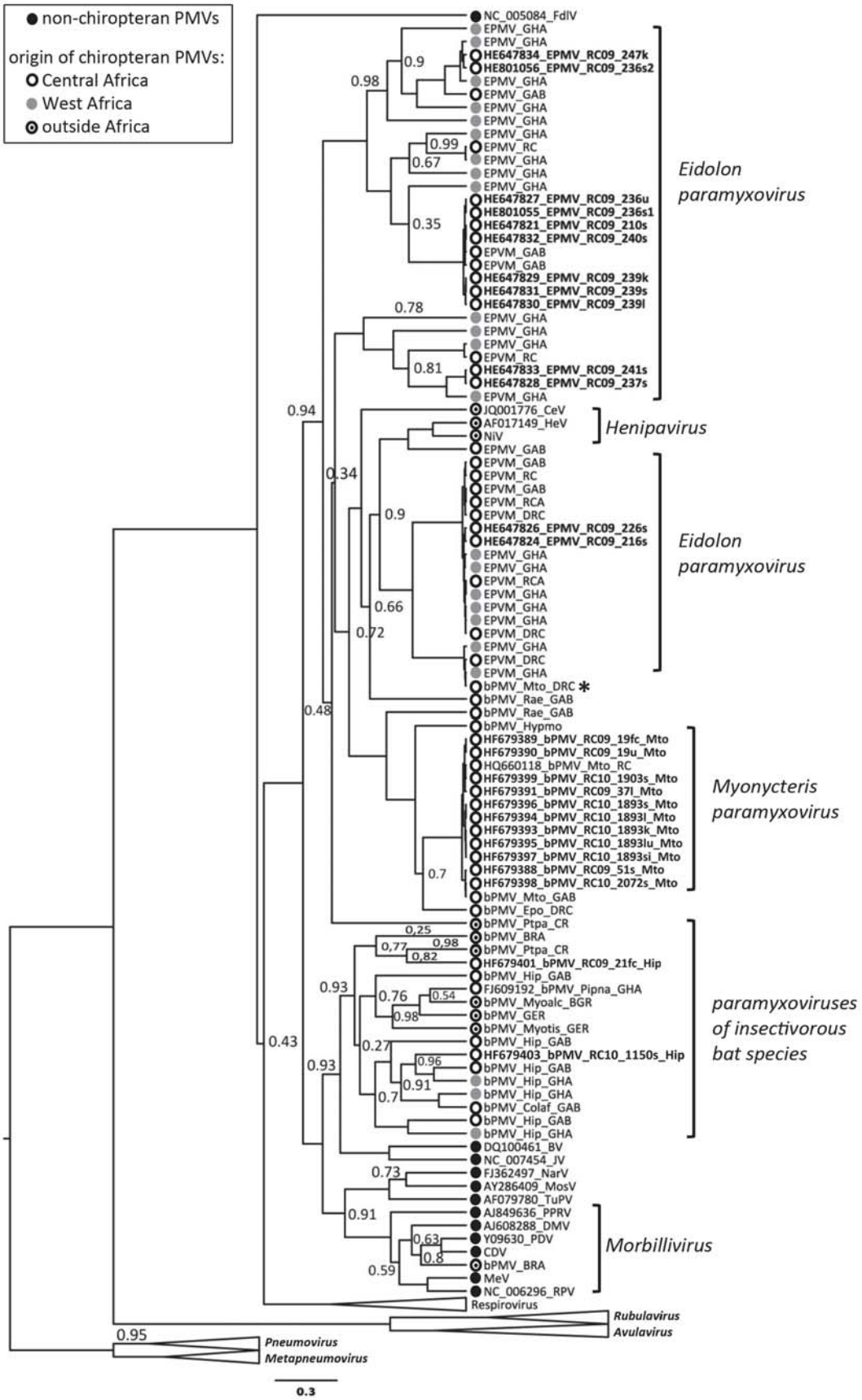


Figure 5.6: Phylogenetic tree showing the placement of Eidolon paramyxovirus (EPMV) sequences in the diversity of *Paramyxoviridae*, based on a partial large gene sequence (473 nt) of the *Paramyxovirinae*-conserved (PAR) fragment. Trees were computed by using both, Bayesian and maximum likelyhood (ML) frameworks based on the general time reversible nucleotide substitution model with gamma distributed rate heterogeneity and invariant sites (GTR+G+I). Bayesian analysis was computed with BEAST version 1.7.1 (http://beast.bio.ed.ac.uk/Main_ Page) under the assumption of a relaxed, uncorrelated lognormal clock and the Yule process speciation model. Values given are posterior probabilities (above branches) and values resulting from nonparametric bootstrapping (below branches; 500 pseudoreplicates) after analysis in PhyML version 3.0 (http://www.atgc-montpellier.fr/phyml/). For better visibility, only posterior probabilities values below 1 are indicated. Scale bar indicates nucleotide substitutions per site. Bat paramyxoviruses (bPMV) are printed in grey. Paramyxovirus sequences retrieved in this study are marked printed in bold and named according to the following pattern: virus_ origin and year_ individual field ID and sample. APMV, *Avian paramyxovirus*; AMPV, *Avian metapneumovirus*; bPIV, *Bovine parainfluenza virus*; CDV, *Canine distemper virus*; CedPV, *Cedar paramyxovirus*; EPMV, Eidolon paramyxovirus; FDLV, *Fer-de-lance virus*; HeV, *Hendra virus*; hPIV, *Human parainfluenza virus*; HRSV, *Human respiratory syncytial virus*; JV, *J-virus*; MenV, *Menangle virus*; MoV, *Mossmann virus*; MPRV, *Mapuera virus*; MPV, *Murine pneumonia virus*; NarV, *Nariva virus*; NiV, *Nipah virus*; PPRV, *Peste-des-petits-ruminants virus*; RPV, *Rinderpest virus*; SV, *Sendai virus*; TioV, *Tioman virus*; TPMV, *Tupaia paramyxovirus*.

Sequences from the PAR fragment were not available for all detected viruses since the PCR assay is broader and less sensitive than the HRM-based assay. For the HRM fragment there are also more reference sequences represented on GenBank, so the tree based on this fragment reveals a more detailed picture (figure 5.7). Novel EPMV sequences from Central Africa cluster in three distinct groups interspersed with EPMV sequences from West Africa and the genus *Henipavirus*. Sequences derived from *My. torquata* during this study build a separate group together with other PMVs from the same bat species. There is one exception, a single sequence found in *My. torquata* during another study (Drexler et al. 2012) that groups with EPMV sequences (marked with * in figure 5.7). All myonycterine bats tested positive are *My. torquata* originating from Central Africa. Sequences derived from other fruit bats (*Hypsignathus monstrosus*, *Rousettus aegyptiacus*, *Epomops* sp.) are associated with the abovementioned groups, yet not interspersed. As mentioned above, viruses belonging to the genus *Henipavirus* nest within the diversity of novel PMVs. Looking at the overall tree topology groups built by sequences derived from fruit bats are most closely associated with viruses belonging to the genus *Henipavirus*. PMVs derived from insectivorous bat species, including the two sequences from *Hipposideros* sp. derived during the present study, build a separate group in the phylogenetic tree. This group is most closely associated to two unclassified PMVs from rodents, *Beilong virus* and *J-virus*. The latter are sufficiently different from all classified PMVs to have been proposed to build a new genus (Magoffin et al. 2007). Together they form a sister-clade to morbilliviruses, which contain viruses from various host species including the human pathogenic measles viruses.

Host switch by paramyxoviruses The great variety of novel PMV sequences from bats raises the question about the host origin of PMVs circulating to date, and the importance of

Figure 5.7 *(following page)*: Phylogenetic tree showing the placement of novel paramxovirus (PMV) sequences in the diversity of *Paramyxoviridae*, based on a partial large gene sequence (461 nt) of the *Henipa-*, *Morbilli-*, *Respirovirus*-conserved (HRM) fragment. PMV sequences retrieved in this study are printed in bold and named according to the following pattern: virus_ origin and year_ individual field ID and sample_ species. Abbreviations are specified below. Circles indicate origin: grey = Central Africa; open = West Africa; open with black dot = South America, Middle America, Asia, or Europe. PMVs from other species than bats are marked with black circles, irrespective of their spatial origin. Trees were computed by using BEAST version 1.7.1 (http: beast.bio.ed.ac.uk Main_ Page) under the assumption of a relaxed, uncorrelated lognormal clock and the Yule process speciation model. Analyses are based on the general time reversible nucleotide substitution model with gamma distributed rate heterogeneity and invariant sites (GTR+G+I). Values given are posterior probabilities (pp). For better visibility, only pp values below 1 are indicated. Scale bar indicates nucleotide substitutions per site. Full tree and accession numbers are given in A.4. Virus abbreviations: bPMV, bat paramxovirus; BV, *Beilong virus*; CDV, *Canine distemper virus*; CeV, *Cedar virus*; DMV, *Dolphin morbillivirus*; FdLV, *Fer-de-Lance virus*; HeV, *Hendra virus*; JV, *J-virus*; MeV, *Measles virus*; MosV, *Mossman virus*; NiV, *Nipah virus*; NarV, *Nariva virus*; PDV, *Phocine distemper virus* PPRV, *Peste-des-petits-ruminants virus*; RPV, *Rinderpest virus*; TuPV, *Tupaia virus*. Country abbreviations: BRA, Bazil; CR, Costa Rica; DRC, Democratic Republic of Congo; GAB, Gabon; GER, Germany; GHA, Ghana; RC, Republic of Congo; RCA, Republic Central Africa. Species abbreviations: Colaf, *Coleura afra*; Epo, *Epomophorus* sp.; Hip, Hipposideridae; Hypmo, *Hypsignathus monstrosus*; Mto, *Myonycteris torquata*; Myoalc, *Myotis alcathoe*; Myotis, *Myotis* sp.; Pipna, *Pipistrellus nanus*; Ptpa, *Pteronotus parnellii*; Rae, *Rousettus aegyptiacus*. Sample abbreviations: s, spleen; l, liver; k, kidney; lu, lung; si, small intestines; fc, feces; u, urine.


non-chiropteran PMVs
origin of chiropteran PMVs:
Central Africa
West Africa
outside Africa
NC_005084_FdlV
EPMV_GHA
EPMV_GHA
HE647834_EPMV_RC09_247k
HE801056_EPMV_RC09_236s2
EPMV_GHA
EPMV_GAB
EPMV_GHA
EPMV_GHA
EPMV_GHA
EPMV_RC
EPMV_GHA
EPMV_GHA
EPMV_GHA
HE647827_EPMV_RC09_236u
HE801055_EPMV_RC09_236s1
HE647821_EPMV_RC09_210s
HE647832_EPMV_RC09_240s
EPVM_GAB
EPVM_GAB
HE647829_EPMV_RC09_239k
HE647831_EPMV_RC09_239s
HE647830_EPMV_RC09_239l
EPMV_GHA
EPMV_GHA
EPMV_GHA
EPVM_RC
HE647833_EPMV_RC09_241s
HE647828_EPMV_RC09_237s
EPVM_GHA
Eidolon paramyxovirus
JQ001776_CeV
AF017149_HeV
NiV
Henipavirus
EPMV_GAB
EPVM_GAB
EPVM_RC
EPVM_GAB
EPVM_RCA
EPVM_DRC
HE647826_EPMV_RC09_226s
HE647824_EPMV_RC09_216s
EPMV_GHA
EPMV_GHA
EPVM_RCA
EPVM_GHA
EPMV_GHA
EPMV_GHA
EPVM_DRC
EPMV_GHA
EPVM_DRC
EPMV_GHA
Eidolon paramyxovirus
bPMV_Mto_DRC *
bPMV_Rae_GAB
bPMV_Rae_GAB
bPMV_Hypmo
HF679389_bPMV_RC09_19fc_Mto
HF679390_bPMV_RC09_19u_Mto
HQ660118_bPMV_Mto_RC
HF679399_bPMV_RC10_1903s_Mto
HF679391_bPMV_RC09_37l_Mto
HF679396_bPMV_RC10_1893s_Mto
HF679394_bPMV_RC10_1893l_Mto
HF679393_bPMV_RC10_1893k_Mto
HF679395_bPMV_RC10_1893lu_Mto
HF679397_bPMV_RC10_1893si_Mto
HF679388_bPMV_RC09_51s_Mto
HF679398_bPMV_RC10_2072s_Mto
Myonycteris paramyxovirus
bPMV_Mto_GAB
bPMV_Epo_DRC
bPMV_Ptpa_CR
bPMV_BRA
bPMV_Ptpa_CR
HF679401_bPMV_RC09_21fc_Hip
bPMV_Hip_GAB
FJ609192_bPMV_Pipna_GHA
bPMV_Myoalc_BGR
bPMV_GER
bPMV_Myotis_GER
bPMV_Hip_GAB
HF679403_bPMV_RC10_1150s_Hip
bPMV_Hip_GAB
bPMV_Hip_GHA
bPMV_Hip_GHA
bPMV_Colaf_GAB
bPMV_Hip_GAB
bPMV_Hip_GHA
paramyxoviruses of insectivorous bat species
DQ100461_BV
NC_007454_JV
FJ362497_NarV
AY286409_MosV
AF079780_TuPV
AJ849636_PPRV
AJ608288_DMV
Y09630_PDV
CDV
bPMV_BRA
MeV
NC_006296_RPV
Morbillivirus
Respirovirus
Rubulavirus
Avulavirus
Pneumovirus
Metapneumovirus
0.3

host switch events along paramyxovirus evolution. Based on the analysis of a large sample of phylogenetic trees for which putative ancestral hosts were mapped at each node of all trees, host switch analyses were performed, following a methodology described in Drexler et al. (2012). For this specific analysis, single sequences were removed from the dataset because they were the only PMVs available in their host species. Using a parsimony-based approach, the Bayesian consensus tree was modified to estimate and visualise host switches along branches. When this was applied to the HRM-based tree, the total number summed up to 21 events (figure 5.8).
However, parsimonious approaches favour the scenario that requires the least evolutionary changes to explain the present data and are thus based on tree topology. To account for uncertainty in tree topology, analyses have to be done on a large set of phylogenetic trees, hence host switches were summarised over a total of 11,250 trees and averaged summarised values were calculated for each animal order. Doing so, primates and bats are reflected as the main donors of actual circulating PMVs, with a slight bias of one host switch in average towards primates (figure 5.9, A). When hosts were randomly assigned to sequences and analyses were re-done they revealed a clear bias towards chiropterans. This reflects sampling efforts and therefore indicates that the dataset contained phylogenetic information relevant to the question. When averaged host switches for bats and primates are related to recipients, primates are shown to be primary donors towards bats (chiroptera), birds (aves) and ungulates (cetartiodactyla) while bats are primary donors for carnivores and rodents (figure 5.9, B). When considering all orders as possible donors, primates still remain the main source for chiroptera PMVs and chiroptera, followed by ungulates, being the main donors for primates (data not shown). Overall, most host switches occurred from chiroptera to rodents (3.3 in average) and from primates to chiroptera (3.0 in average). These results were confirmed when a probabalistic approach (likelihood ancestral state reconstruction) was applied on the dataset (data not shown).

Figure 5.8: Schematic visualisation of host switches (grey circles) on consesus phylogenetic tree based on Bayesian analysis of the HRM-fragment (see figure 5.7). Tree tips show respective host order instead of virus species. Most probable PMV donors for subtrees are depicted above branches.

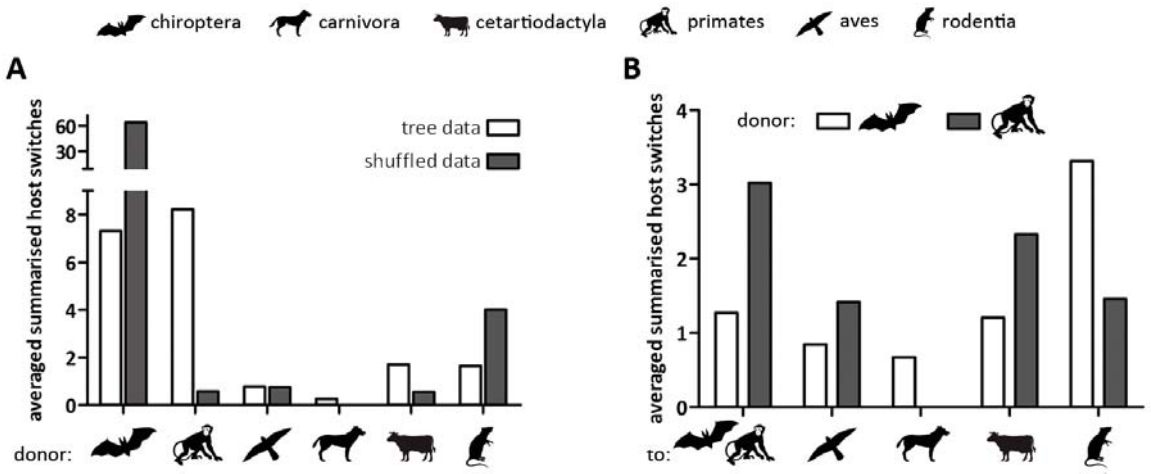


Figure 5.9: Average number of host switches of paramyxoviruses summarised over 11,250 bayesian trees, modelled by parsimony ancestral state reconstruction. A: Host switches summarised per mammalian order. B: Host switches from chiroptera and primates to other mammalian orders.

Isolation of paramyxoviruses Virus isolation was attempted for several samples for which enough appropriate material was available (see table 5.2). Despite the use of various cell lines and embryonated chicken eggs, no virus growth was documented. No CPE was observed after multiple passages and parallel qPCR analyses of supernatant and allantois liquid did not show an increase of viral copy numbers.

5.2.3 Screening for filoviruses

Both, Côte d'Ivoire (RCI) and the Republic of Congo (RC) are known for filovirus haemorrhagic fever outbreaks and bats have been shown to carry and transmit these viruses. Cultivation of filoviruses would require biosecurity level 4 laboratories (BSL4) but routine cell culture screening for the present work was done at BSL3 level only. To be able to work under BSL3 conditions, all individuals of which samples were used in cell culture screenings have been tested for the presence of filoviruses prior to inoculation. Liver samples were tested where available, otherwise blood samples were screened using a real-time PCR and a conventional PCR approach. In total, 358 samples were tested but none gave a positive result in either PCR. Samples were therefore considered safe with respect to filoviruses and were used for inoculation of cells under BSL3 safety conditions.

5.3 Cell culture screening

To identify novel viruses in bats organ samples from animals captured in RC and blood samples from animals from RCI were used to inoculate VeroE6 and R05T cells. No cytopathic effect (CPE) was observed after multiple passages on any cell culture inoculated with organ samples (data not shown). Of the blood samples, 46 pools of 267 individuals belonging to 19 different species were built of which the majority (172 individuals, 23 pools) belong to the species *Myonycteris torquata* (figure 5.10).

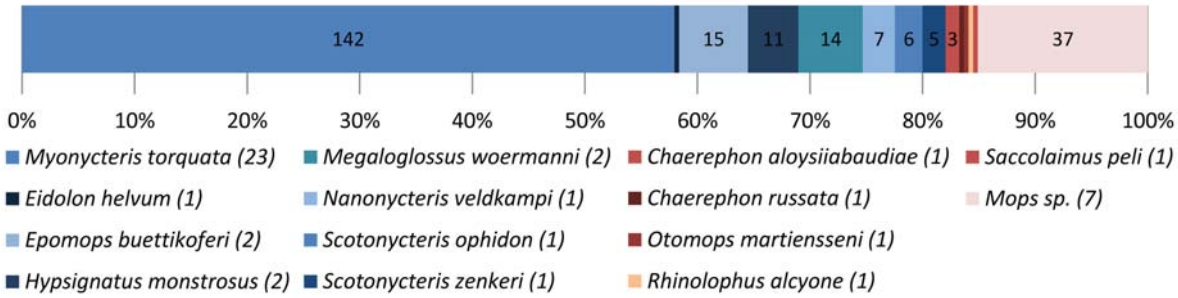


Figure 5.10: Origin of blood samples used for inoculation of cell lines. Distribution of bat species captured in the *Parc National de Taï* (Côte d'Ivoire). Numbers in parenthesis behind species names indicate number of pools built from available blood samples, numbers in bar represent sample number (no number given equals single individuals). Frugivorous species are depicted in blue, insectivorous species in red. *Mops* sp. include 18 *M. thersites*, five *M. leonis*, two *M. spurrelli*, one *M. nanulus*, and one that was not specified on species level.

5.4 Identification and characterization of a novel reovirus

One pool (B30) composed of three blood samples from bats from RCI showed a CPE on Vero cells at day 7 in the second passage. Blood samples originated from three male Duke of Abruzzi's free-tailed bats (*Chaerephon aloysiisabaudiae*). The virus was isolated and used in further analyses.

5.4.1 Molecular characterisation

Random PCR with subsequent sequencing is an efficient way to gain sequence information of unknown viruses. Here, 56 clones were sequenced after PAN-PCR (see section 4.6.6). Of the resulting 112 individual sequences 79 could be assembled to 19 contigs. These contigs and all remaining single sequences were compared to GenBank entries on both nucleotide and protein level. In total, nine contigs and three individual reads showed homologies exclusively with two viruses belonging to the family *Reoviridae*, *Colorado tick fever virus* (CTFV) and *Eyach virus* (EV), both containing 12 genomic dsRNA segments. Details of BLAST results are given in table 5.3.

Table 5.3: BLAST results of *Taï Forest reovirus* (TFRV) against GenBank entries. Virus hits are printed in bold. Numbers are identical sites/ aligned sites (identity). Seq. = sequence; no. = number

Seq.	length [nt]	identities (nt)	identities (aa)
C1	836	Human DNA sequence from clone RP5-882O7; 791/852 (93%)	no hit
C2	540	**EV segment 1, cs; 319/483 (66%) 319/483 (66%)**	**putative RNA-dependent RNA polymerase VP1 [CTFV]; 107/166 (65%)**
C3	636	no hit	**VP2 [EV]; 86/200 (43%)**
C4	883	Waddlia chondrophila 2032/99 annotated genome fragment,clone 1103163150229; 812/864 (93%)	Nef attachable protein [Homo sapiens]; 119/213 (56%)
C5	715	**EV segment 1, cs; 225/337 (67%)**	**RNA-dependent RNA polymerase VP1 [EV]; 121/233 (52%)**
C6	952	Homo sapiens 2 BAC RP11-105E24; 877/976 (90%)	hypothetical protein [Homo sapiens]; 71/136 (52%)
C7	500	no hit	**VP2 [Colorado Tick Fever Virus]; 37/151 (25%)**
C8	696	no hit	**VP3 [EV]; 60/192 (31%)**
C9	515	**EV segment 1, cs; 180/269 (67%)**	**RNA polymerase [CTFV]; 86/164 (52%)**
C10	811	**EV segment 1, cs; 231/330 (70%)**	**RNA-dependent RNA polymerase [EV]; 162/256 (62%)**
C11	421	Pan troglodytes BAC clone RP43-55D2 from chromosome 7,complete sequence; 362/407 (88%)	no hit
C12	532	no hit	no hit
C13	473	no hit	**putative RNA-dependent RNA polymerase VP1 [CTFV]; 60/146 (41%)**
C14	427	no hit	no hit
C15	561	Homo sapiens BAC clone RP11-356H17; 498/539 (92%)	no hit
C16	541	no hit	no hit
C17	417	no hit	no hit
C18	396	**EV segment 9, complete sequence; 71/99 (71%)**	**VP9 [CTFV]; 52/110 (47%)**
C19	302	no hit	no hit
no. 67	396	Neurospora cloning vector pTH1108.2; 92/114 (80%)	**RNA-dependent RNA polymerase VP1 [EV]; 34/60 (56%)**
no. 69	457	Neurospora cloning vector pTH1108.2; 94/115 (81%)	**RNA-dependent RNA polymerase VP1 [EV]; 58/98 (59%)**
no. 82	796	Uncultured delta proteobacterium partial 16S ribosomalRNA,clone 73; 108/120 (90%)	**RNA-dependent RNA polymerase [EV]; 127/189 (67%)**

CTFV was used as reference geonome to map contigs of the novel virus so as to get a possible arrangement. Based on this, sequence gaps were closed with 'out-primers', as schematically shown in figure 5.11. To amplify segment ends RACE PCR was used.

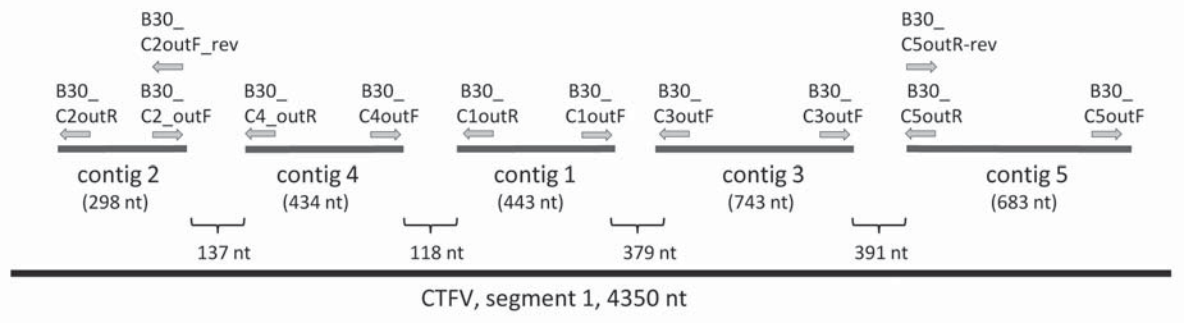


Figure 5.11: Possible arrangement (schematic) of sequence contigs from *Taï Forest reovirus* segment 1 according to *Colorado tick fever virus* (CTFV) reference genome segment 1. Arrows indicate 'out-primers' used for amplification and sequencing to close gaps.

In parallel, RNA was applied to 454 next generation sequencing. Reads were mapped to CTFV and existing Sanger sequences, resulting in four final contigs with sufficient identity to known sequences. To date, sequences are available for segments 1, 2, 3 and 9 of the novel virus (as given in appendix A.6). The novel virus was tentatively named *Taï Forest reovirus* (TFRV) because it was isolated from an animal captured in the *Parc National de Taï* in Côte d'Ivoire. Identification of the detected virus isolate as a reovirus was supported by confirming the presence of a double stranded RNA genome (figure 5.12) and electronmicroscopic pictures (see figure 5.13).

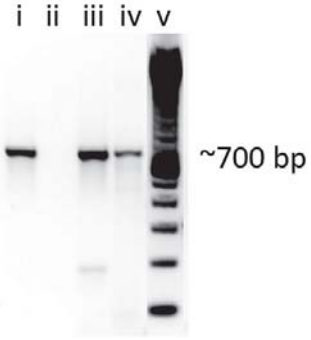


Figure 5.12: Amplicons of B30 on an agarose gel. Templates were i) cDNA made with R6 primers, ii) cDNA made without reverse transcriptase, iii) cDNA made with gene-specific forward primer, and iv) cDNA made with gene-specific reverse primer. Lane v) shows the size marker (100bp).

Based on sequence information of segment 1, a qPCR assay was designed to determine viral load and to specifically re-screen all the bat blood samples from PNT. While none of the other bat species were tested positive for TFRV, all three individuals contained in the pool were tested positive. Copy numbers were 7.6 x 10^3, 2.6 x 10^4, and 2.1 x 10^5 genome equivalents per ml.
When assembled segments were blasted they revealed amino acid (aa) identities between 28%

and 56% to CTFV and EV, respectively (table 5.4). Subsequently, segment sequences were blasted against all reovirus GenBank entries except coltiviruses, which resulted in identification of only very short alignable sequence stretches (max. 45 nucleotides/ 155 aa; data not shown). Alignments were computed with various algorithms (clustalW, muscle, mafft) for all open reading frames (ORFs) of polymerase genes of ICTV listed reovirus species available on GenBank (Larkin et al. 2007; Katoh et al. 2009; Edgar 2004). However, when compared to all reoviruses but coltiviruses, TRFV did not exhibit identity levels above 26% and 12% for nucleotide (nt) and amino acid characters, respectively (data not shown). Since such identity levels do not deviate much from random expectations (25% and 5% for nt and aa characters, respectively), it was impossible to ascertain that polymerase genes from all reoviruses are homologous. Accordingly, it was not possible to run phylogenetic analyses on this alignment, since phylogenetic analyses rely on primary homology.

Table 5.4: Identity levels of *Taï Forest reovirus* (TFRV) and coltivirus segments on amino acid (aa) level, and allocation of putative protein functions based on known proteins according to UniProt database. Numbers are identical aa/ mapped aa (identity).

Segment	length [nt]	identity aa-level CTFV	EV	putative gene function	UniProt
1	4381	816/1435 (56%)	811/1431 (56%)	RNA-dependent RNA polymerase	Q9DSQ0
2	3882	420/1095 (38%)	452/1212 (37%)	RNA-methyltransferase	Q9ENL4
3	2063	188/665 (28%)	191/639 (29%)	membrane protein	Q9ENL3
9	1099	142/360 (39%)	138/360 (38%)	structural protein (viral capsid)	O93214

With few exceptions, each reoviral segment contains one long ORF coding for a single protein, flanked by non-coding regions and conserved terminal sequence motifs. So far, only the 5'ends of segments 1 and 2 have been sequenced. According to these results TFRV shows a 5'-AwUAAUGU consensus sequence at the 5'end. One possible long ORF was identified for each of the available segments from TFRV (S1, S2, S3, and S9) with the NCBI ORF finder. S9 of coltiviruses build an exception since they contain an Opal stop codon, followed by a cytosine residue (Attoui et al. 2000) which allows for a read-through phenomenon resulting in incorporation of arginine, cysteine or tryptophan instead (Feng et al. 1990; Strauss & Strauss 1994). Thus, coltivirus S9 codes for two proteins beginning with the same start codon, a shorter viral protein 9 (VP9) and a longer VP9' protein (Attoui et al. 2002). When comparing ORF lengths and positions of start codons, TFRV segments and ORFs seem to be longer than those of CTFV and EV, at least for S1 and S2 (table 5.5). However, since the 3'ends are still missing for all segments, no definite protein length can be stated yet for TFRV. For S3 and S9 also the 5'ends are yet to be sequenced and it is likely that the full sequence of S3 will reveal another start codon further upstream. However, this is unlikely for S9 since one ORF has been identified in TFRV that matches in length and starting position to the VP9 ORF of CTFV and EV (table 5.5).

Table 5.5: Comparison of nucleotide (nt) lengths and nt position (in parentheses) of the starting codon of open reading frames (ORF) of the novel *Taï Forest reovirus* (TFRV), *Colorado tick fever virus* (CTFV) and *Eyach virus* (EV).

	ORF length [nt] (start nt position)		
Segment	**TFRV**	**CTFV**	**EV**
S1	4370 (12)	4308 (14)	4308 (13)
S2	3848 (35)	3630 (46)	3828 (45)
S3	1986 (78*)	3549 (12)	3549 (11)
S9	1008 (29*)	1014 (41)	1014 (41)

*5'end not amplified yet

Possible gene functions can be predicted based on homology to CTFV and the according Pfam and UniProtKB entries for this virus (table 5.5) (The Uniprot Consortium 2012; Finn et al. 2010). VP1 of TFRV is aligned to RNA polymerase domains (Pfam-B entry PB010949), hence, as in CTFV and EV, the first segment of TFRV probably codes for the RNA-dependent RNA polymerase. Despite the absence of a formal proof of sequence homology among reovirus *pol* genes, two functional motifs are present in all reoviruses (Attoui et al. 2002): motif SG (positions 754-755 for CTFV and EV, positions 753-754 in TFRV) and motif GDD (positions 816-181 for CTFV, 815-817 for EV, and 814-816 for TFRV). According to UniProt entries, VP2 on S2 might code for a RNA methyltransferase, VP3 on S3 for a membrane protein, and the short ORF VP9 on S9 for a capsid protein, but sequence homology with other (reo-)viruses is too low to assign a clear biological function to the other segments.

5.4.2 Cell culture characterisation

To visualise viral particles infected cells were formalin-fixed at onset of the cytopathic effect (CPE) three days after infection and ultra-thin sections were prepared. Electronmicroscopic pictures showed particles of approximately 70 nm in diameter and typical inner and outer icosahedral capsids (figure 5.13). These are characteristic for reoviruses.

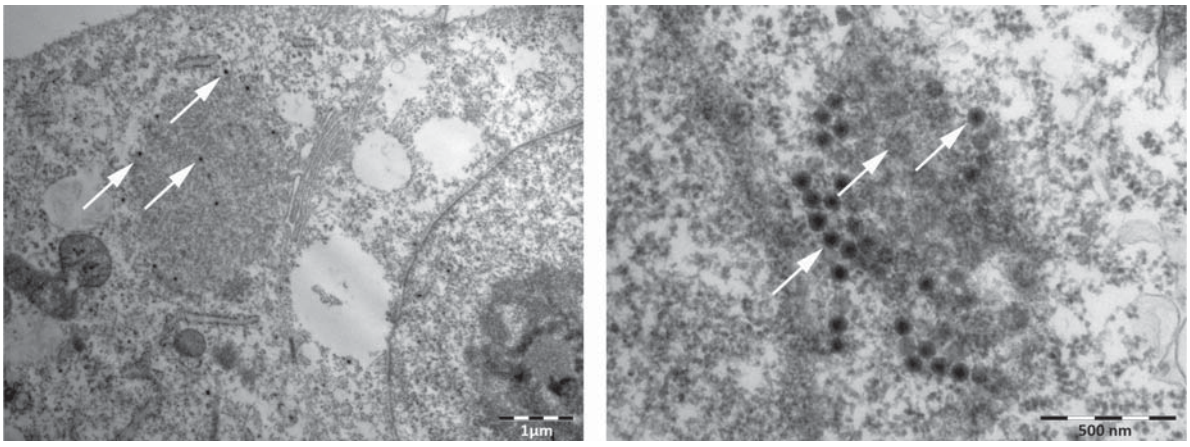


Figure 5.13: Ultra-thin sections of Vero cells infected with *Taï Forest reovirus* (TFRV) at 3 days post infection reveal reovirus-characteristic particles (white arrows) with typical inner and outer icosahedral capsids of approximately 70 nm in diameter. Photos taken by A.Kurth.

To estimate the possible host range of TFRV it was used for inoculation of various cell lines. The virus was able to induce a CPE on C6/36 insect cells and on various mammalian cell lines (figure 5.15): Primate kidney cells (VeroE6), a fruit bat cell line originating from *Rousettus aegyptiacus* (R05T), and two human cell lines, lung fibroblasts (MRC-5) and liver cells (Hep2).
Copy numbers determined with qPCR do only give the number of genome equivalents in a given solution. To determine the amount of infectious particles virus stocks were titrated. On primate cells (VeroE6) the highest titer is reached three days post infection (dpi) with 3.16 x 10^4 per ml. Despite reduction of infectious particles in the cell culture after day 3 p.i., the number of genomic equivalents is still rising until day 8 p.i. (end of experiment; figure 5.14). When subjected to ether, virus particles did not show reduced infectivity (data not shown) which has been described before for EV (Rehse-Küpper et al. 1976).

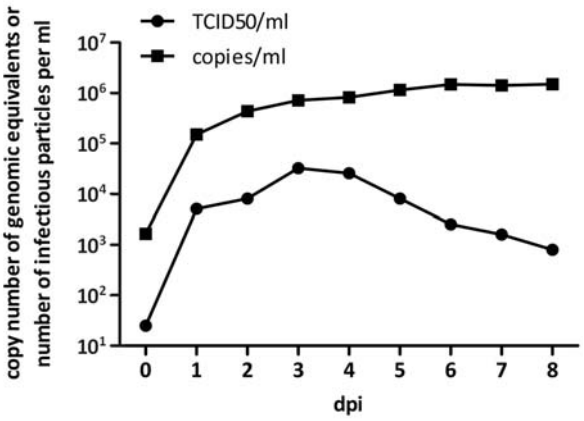


Figure 5.14: Growth kinetics of *Taï Forest Reovirus* (TFRV) on VeroE6 cells. A 24-well tissue culture plate was infected with TFRV and one aliquot was harvested each day from 0 to 8 days post infection (dpi). Cleared supernatant was extracted and used for cDNA synthesis and specific real-time PCR, resulting in copy number of genomic equivalents per ml. In parallel, supernatant was used for titration, giving the number of infectious particles (TCID50) per ml.

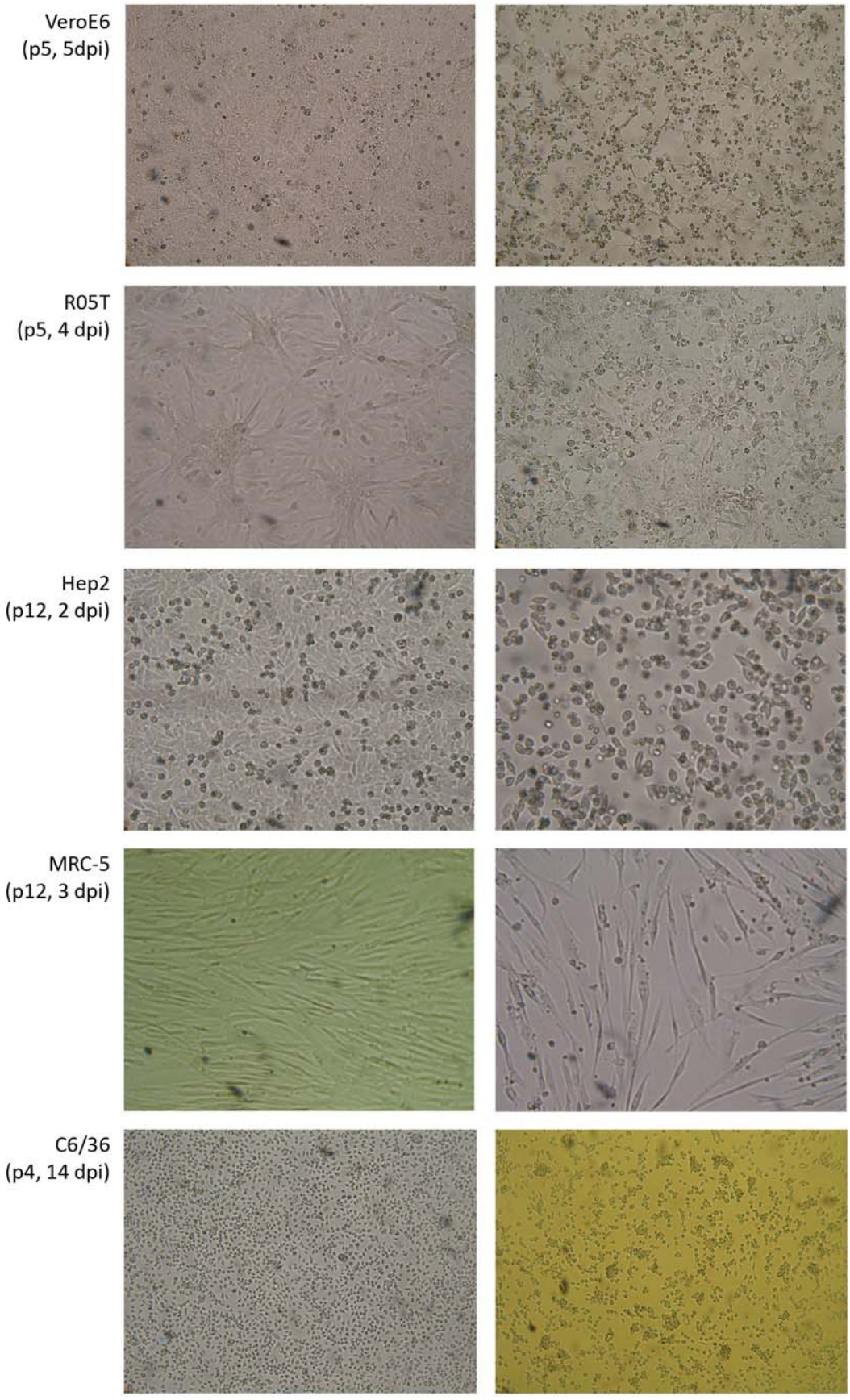


Figure 5.15: Cells infected with TFRV (right side) and uninfected control cells (left side). p, passage; dpi, days post infection.

5.5 Filovirus serology

To estimate the impact of using whole blood versus serum for serological analyses, initially negative bat whole blood samples were spiked with an anti-Marburg goat serum and tested in an antibody capture ELISA. It was shown that the optical density (OD) signal was not impaired by whole blood compared to clean serum (figure 5.16).

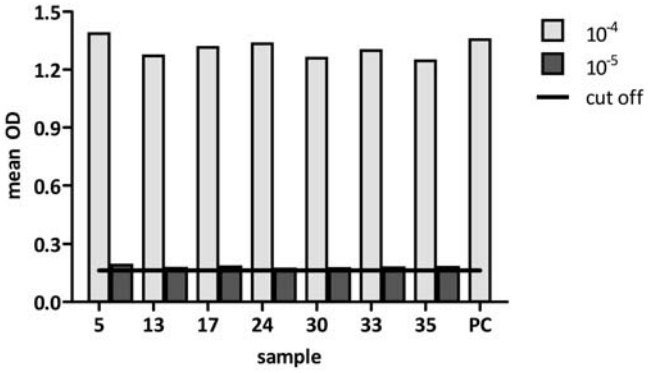
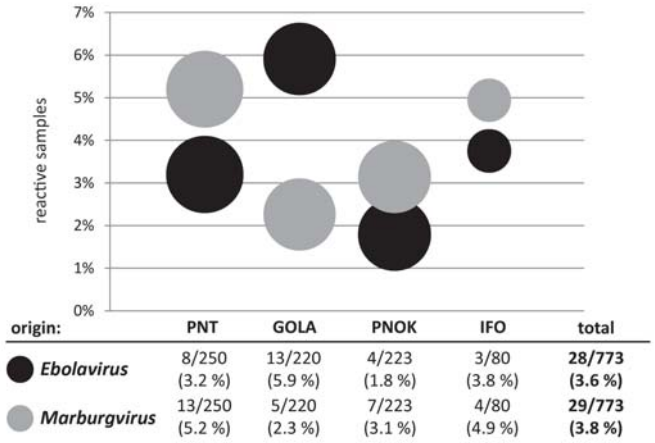


Figure 5.16: Impact of using whole blood vs. serum on ELISA results. Initially seronegative bat blood samples were spiked with two dilutions (10^{-4} and 10^{-5}) of positive control (PC) serum and tested for reactivity with cell lysate of *Marburgvirus* infected cells. Values of 10^{-5} vary between 0.171 and 0.191; cut off value is 0.163.

Consecutively, all available bat blood samples (n = 773) have been screened for IgG antibodies against *Ebola-* (EBOV) and *Marburgvirus* (MARV) by ELISA. In a conservative approach as described in section 4.7.3, 28 samples (3.6%) showed reactivity against EBOV antigen and 29 (3.8%) against MARV antigen (figure 5.17). For details on tested bat species please refer to appendix A.5.



origin:	PNT	GOLA	PNOK	IFO	total
Ebolavirus	8/250 (3.2 %)	13/220 (5.9 %)	4/223 (1.8 %)	3/80 (3.8 %)	**28/773 (3.6 %)**
Marburgvirus	13/250 (5.2 %)	5/220 (2.3 %)	7/223 (3.1 %)	4/80 (4.9 %)	**29/773 (3.8 %)**

Figure 5.17: Results of filovirus-antibody screening. Samples were tested in an antibody-capture ELISA for binding with cell lysates of *Ebola-* (EBOV) and *Marburgvirus* (MARV) infected cells. Size of circles corresponds to number of tested samples; number of positive tested/ total number tested; % give proportion of samples tested positive. PNT, *Parc National de Taï*, Côte d'Ivoire; PNOK, *Parc National d'Odzala-Kokoua* (including samples from Brazzaville), Republic of Congo; IFO, *Industrie Forestière d'Ouesso*, Republic of Congo; GOLA, *Trans-boundary Peace Park*, Sierra Leone and Liberia.

Reactive samples originated from three insectivorous bat genera (*Hipposideros* sp. *Mops* sp., and *Nycteris* sp.) and eight frugivorous bat species (*Epomops franqueti*, *Epomops buettikoferi*, *Hypsignathus monstrosus*, *Megaloglossus woermanni*, *Micropteropus pusillus*, *Myonycteris torquata*, *Myonycteris leptodon*, and *Rousettus aegyptiacus*) (table 5.6). Twelve samples have been reactive for both, EBOV and MARV. These originate from three *E. buettikoferi*, six *H. monstrosus*, two *R. aegyptiacus*, and one *Mops thersites*. A two-sided Fisher's exact test revealed no influence of age (p-values 1.00 and 0.55 for EBOV and MARV, respectively) or gender (p-values 0.08 and 0.26) on the outcome of the ELISA test.

Table 5.6: Bat species with antibodies showing reactivity with filovirus antigens. Samples were tested in an antibody-capture ELISA for binding with cell lysates of *Ebola-* (EBOV) and *Marburgvirus* (MARV) infected cells. Number of reactive samples in ELISA/ number of tested samples per capture site. PNT, *Parc National de Taï*, Côte d'Ivoire; PNOK, *Parc National d'Odzala-Kokoua* (including samples from Brazzaville), Republic of Congo; IFO, *Industrie Forestière d'Ouesso*, Republic of Congo; GOLA, *Transboundary Peace Park*, Sierra Leone and Liberia. n.a. = not available; *insectivorous bat species.

genus	**capture site**					
MARV	**PNT**	**GOLA**	**IFO**	**PNOK**	**total**	**total (%)**
Epomops	4/15	0/14	0/12	2/21	**6/62**	**9.7**
Hypsignathus	2/11	5/23	n.a.	4/36	**11/70**	**15.7**
Megaloglossus	1/14	0/13	0/1	0/7	**1/35**	**2.9**
*Mops**	0/26	0/4	1/3	0/4	**1/37**	**2.7**
Myonycteris	6/159	0/24	1/52	0/72	**7/307**	**2.3**
*Nycteris**	n.a.	0/8	0/2	1/1	**1/11**	**9.1**
Rousettus	n.a.	n.a.	2/2	n.a.	**2/2**	**100.0**
EBOV						
Epomops	4/15	1/14	0/12	0/21	**5/62**	**8.1**
*Hipposideros**	n.a.	4/91	0/4	0/3	**4/98**	**4.1**
Hypsignathus	2/11	6/23	n.a.	3/36	**11/70**	**15.7**
Micropteropus	n.a.	n.a.	n.a.	1/40	**1/40**	**2.5**
*Mops**	0/26	1/4	1/3	0/4	**2/37**	**5.4**
Myonycteris	2/159	1/24	0/52	0/72	**3/307**	**1.0**
Rousettus	n.a.	n.a.	2/2	n.a.	**2/2**	**100.0**

Since ELISA assays are prone to detect unspecific binding, 38 reactive samples were analysed by immunoblotting for confirmation and only those samples were considered truly positive, that reacted with at least one filovirus protein. Ten samples from PNOK (four and six for EBOV and MARV, respectively) could not be further analysed due to a lack of sufficient suitable material. Overall, about half (42% EBOV, 63% MARV) of the samples reactive in ELISA could be confirmed in Western blot. None of the MARV-reactive samples from PNOK were confirmed but all from GOLA and IFO (figure 5.19). Neither for EBOV nor for MARV there was any correlation between intensity of the OD signal and whether or not the result was confirmed in Western blot.

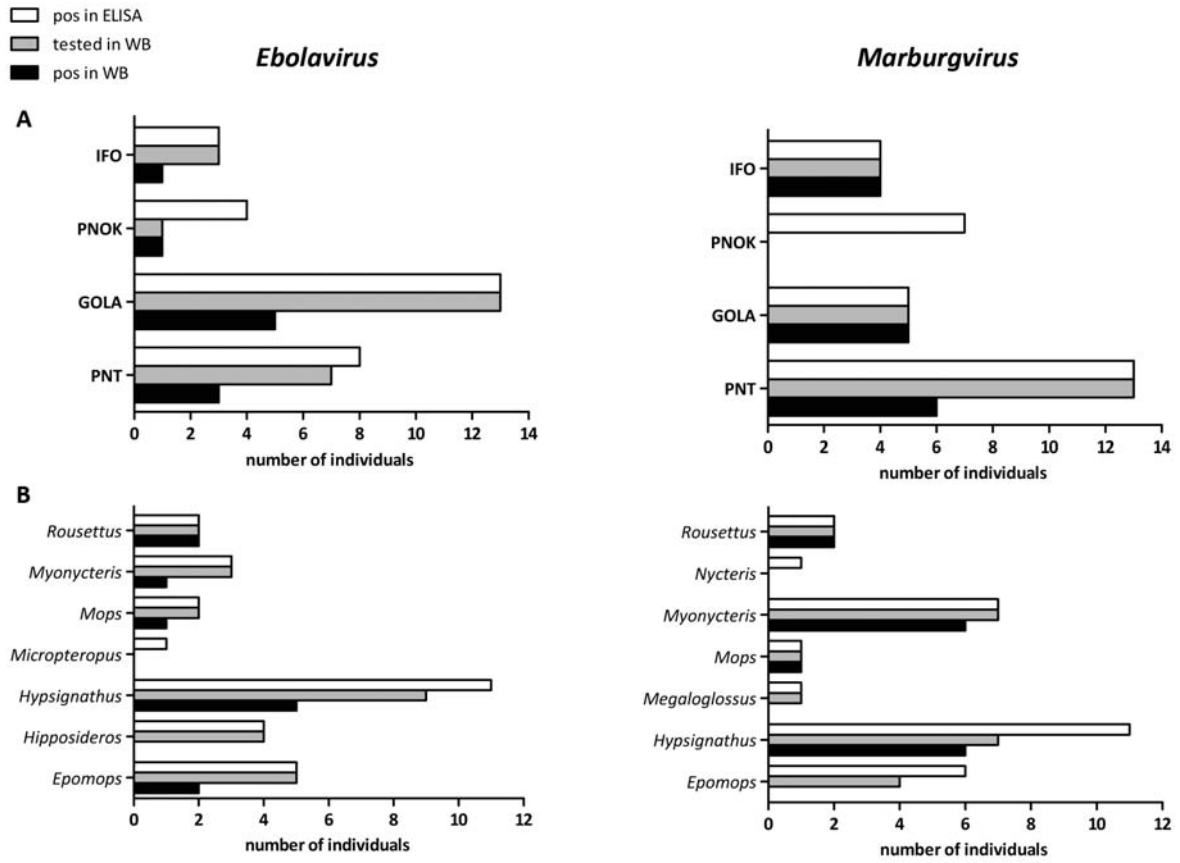


Figure 5.18: Relation between samples reactive in ELISA, tested in Western blot (WB) and positive in WB for *Ebola-* and *Marburgvirus*. A, sorted by field site; B, sorted by bat genus. PNT, Parc National du Taï, Côte d'Ivoire; PNOK, Park National d'Odzala-Kokoua (including samples from Brazzaville), Republic of Congo; IFO, Industrie Forestière d'Ouesso, Republic of Congo; GOLA, Trans-boundary Peace Park, Sierra Leone and Liberia.

For *Ebolavirus*, 24 samples were tested of which ten (41.7%) showed specific bands in Western blot (figure 5.19 A). Four samples (1183, 1185, 32, 833) bound to the nucleoprotein (NP, ∼100 kDa), another four samples (1295, 1350, 968, 1979) to the viral protein 40 (VP40, ∼40 kDa), and two samples (1352, 829) reacted with both proteins. Positive samples originate from one *Mops* cf. *condylurus*, one *My. torquata* (1/307; 0.3%), one *R. aegyptiacus* (1/2; 50%), two *E. buettikoferi* (2/62; 3.2%), and five *H. monstrosus* (5/70; 7.1%), adding up to 1.3% of all tested samples being confirmed reactive against EBOV antigen in Western blot.

For *Marburgvirus* 24 samples were tested of which 15 (62.5%) showed specific bands (figure 5.19 B). Four samples (940, 937, 812, 782) bound to NP only, while eight samples (1887, 1866, 1170, 1350, 1185, 1347, 1345, 1879) showed clear binding to VP30, VP35, and/ or VP40, albeit to a lesser extent also to NP. Due to their similar size (28/ 32/ 38 kDa) binding to any of these viral proteins (VP) cannot readily be distinguished from one another. The remaining three samples (758, 884, 1878) showed equal reactivity against all of these proteins (figure 5.19, B). Positive samples originate from one insectivorous bat species (*Mops thersites* [1/21]) and three fruit bat species: *My. torquata* (6/307; 2%), *H. monstrosus* (6/70; 8.6%), *R. aegyptiacus* (2/2; 100%).

These add up to a total of 1.9% of tested samples being reactive against MARV antigen in Western blot.

Of five samples that were tested for both, EBOV and MARV-directed antibodies, three (1879, 1185, 1350) reacted against both virus antigens in Western blot. While two of these (samples 1879 and 1350, *R. aegyptiacus* and *H. monstrosus*) showed reactivity towards VP40 for both, EBOV and MARV, sample 1185 (*H. monstrosus*) was binding to the NP protein of EBOV, but to not of MARV, and vice versa for the VP40 protein (strong band with MARV antigen, but not with EBOV). None of the samples reacted with the glycoprotein.

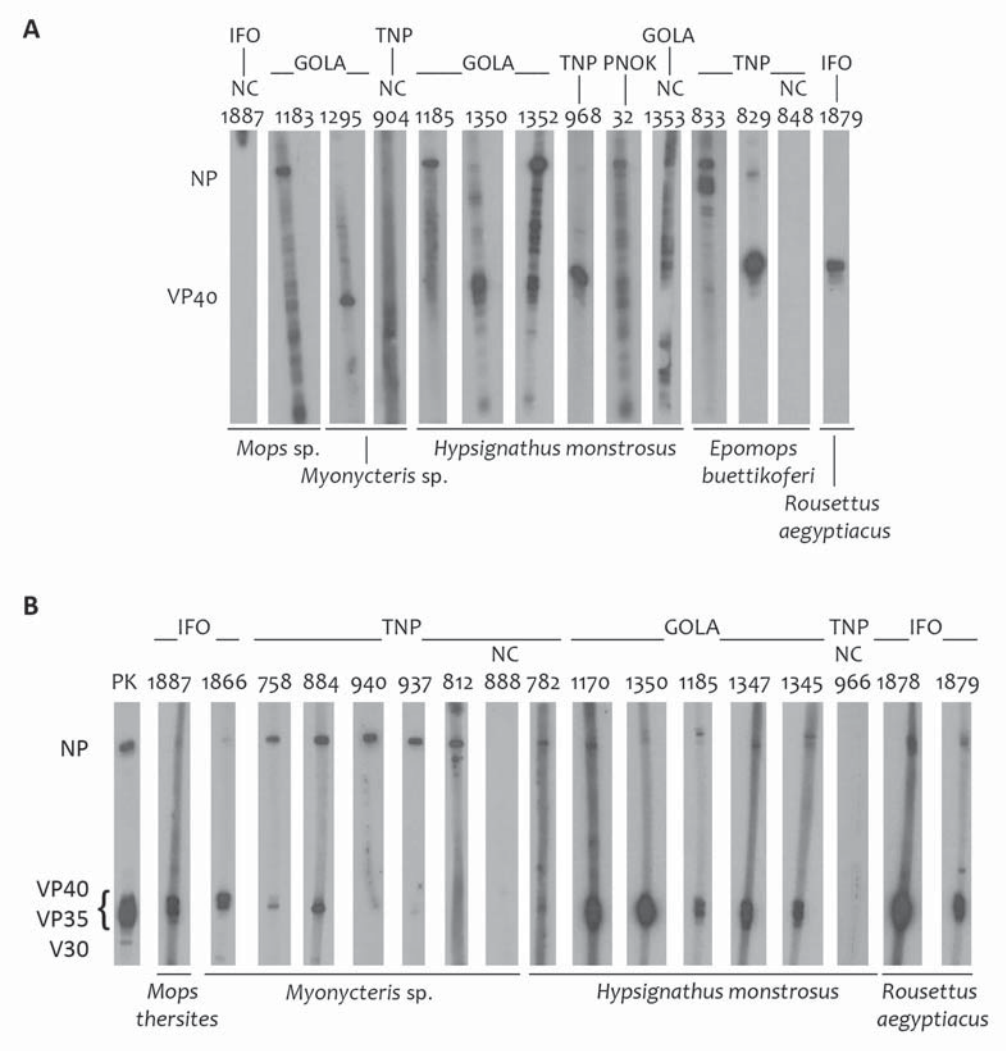


Figure 5.19: Western blots of bat blood samples reacting with (A) *Zaire ebolavirus* (ZEBOV) and (B) *Marburgvirus* (MARV) strain Musoke virus protein. Samples (numbered) were tested for antibodies reacting with full virus protein of MARV or ZEBOV (for *Rousettus aegyptiacus*) or cell lysate of infected cells (ZEBOV except samples 829, 848, 1879). *Mops* sp. (A) include *M. thersites* (1887) and *Mops cf. condylurus* (1183). NC, negative control; TNP, Parc National du Taï, Côte d'Ivoire; PNOK, Park National d'Odzala-Kokoua (including samples from Brazzaville), Republic of Congo; IFO, Industrie Forestière d'Ouesso, Republic of Congo; GOLA, Trans-boundary Peace Park, Sierra Leone and Liberia.

6 Discussion

Aim of the present study was the identification of novel, potentially zoonotic pathogens in free-ranging African bats. Detected viruses should be further characterised and classified to better understand the ecology of these viruses in bats, and to possibly identify candidates likely to cross-species barriers in the future.
The results obtained from the present work contribute to the current knowledge on (emerging) pathogens harboured by African bat species. They are based on samples from free-ranging animals that were captured by myself and co-workers during several field trips in sub-Saharan Africa. Most of the field sites are located in very remote areas in tropcial rainforests, providing major logistic challenges and hence certain limitations to the study: Capturing wild bats is relatively complex, elaborate, and expensive since it requires trained personnel and specialised equipment. It is therefore impossible to conduct temporally continious sampling, so samples are available only from restricted time frames. All material needs to be transported to field sites, which is often only possible by foot or boat. This limits sample storage capacity and does frequently not allow for storage of multiple aliquots. For most applications, sample quality is best if samples are immediately frozen. Liquid nitrogen, however, is not available in every country. The majority of animals was not euthanised but only sampled for throat swabs, excreta, and blood. Obviously, blood yield is limited by animal size and can range from several drops only to few hundred microliters for larger animals. These limitations in material and equipment do not, for example, allow for preparation of serum, but also impede certain analyses that require much of the original or freshly prepared material.

6.1 Detection and characterisation of emerging RNA viruses in bat samples

To identify novel, potentially zoonotic pathogens in wild African bat species, samples were screened with various PCR systems for virus families with known zoonotic potential. The specific question of filovirus distribution and spread was adressed by additional serological screenings. Finally, a selected set of samples was used to inoculate cell cultures so as to attempt virus isolation of previously PCR detected viruses and to isolate novel viruses.

6.1.1 Hantavirus in African bat

Various samples of numberous bat species (see table A.1 for details) were tested for the presence of hantavirus RNA. Using PCR, one sample, stemming from a hairy slit-faced bat (*Nycteris hispida*), was tested positive for a formerly unknown hantavirus, *Magboi virus* (MGBV), which represented the first bat borne hantavirus (see also Weiss et al. 2012b). The presented bat-associated sequence is distinct from other hantaviruses, suggesting association with a novel natural host. This is supported by detection of the virus exclusively in one organ and the absence of histopathological changes, which is pointing towards a persistent infection that is typically observed in natural hosts of hantaviruses (Schönrich et al. 2008). Even though unlikely, a spillover infection from another, yet unrecognized reservoir cannot be ruled out. The theory of an extended host range is supported by description of more hantaviruses in different bat species: *Mouyassué virus* was detected in a banana pipistrelle (*Neoromicia nanus*) from Côte d'Ivoire (Sumibcay et al. 2012), and *Huangpi virus* and *Longquan virus* were found in *Pipistrellus abramus* and *Rhinolophus* sp., respectively, in China (Guo et al. 2013).

While rodents (Rodentia) have long been seen as the only reservoir for hantaviruses this view changed dramatically when first hantaviruses were described in shrews (Soricomorpha, family Soricidae) and moles (Soricomorpha, family Talpidae), and it has been proposed that soricomorphs, not rodents were the original mammalian hosts (Carey et al. 1971; Xiao et al. 1994; Arai et al. 2008; Klempa et al. 2007; Kang et al. 2009, 2011). Bats (Chiroptera) and Soricomorpha belong to the same superorder Laurasiatheria and thus are evolutionary more closely related to each other than to rodents (Nikaido et al. 2001), that do not belong to this superorder. Having this in mind and given the vast variety of viruses that are known to be harboured by chiroptera (Calisher et al. 2006), it is not surprising, that bats also harbour hantaviruses.

Hantavirus cardiopulmonary syndrome (HCPS) caused by new world hantaviruses, displays a strikingly different pathogenicity than old-world hantavirus-caused haemorrhagic fever with renal syndrome (HFRS) (Jonsson et al. 2010; Krüger et al. 2011). It might well be that if bat-associated viruses indeed turn out to be pathogenic for humans they possibly cause a disease quite different from HCPS or HFRS, which might be overlooked by clinicians and not be detected with routine hantavirus diagnostics. At least for shrew-borne hantaviruses cross-reactivity with rodent-borne virus antigens is very limited (Chu et al. 1995; Song et al. 2007), hampering human serodiagnostics. While the ecology of hantaviruses is very well studied in Europe and the Americas, nothing is really known about the situation in Africa. To date, there are only very few reports about hantavirus infections and only one case of HFRS reported from Africa (Coulaud et al. 1987; Klempa et al. 2010). A seroepidemiological study conducted with refined methods in Guinea showed that patients suffering from fever of unknown origin showed higher seroprevalence of hantavirus-specific antibodies (4.4%) in comparison with the general population in the region (1.1%) (Klempa et al. 2010). Yet, when discussing the occurrence of HFRS in Africa, inefficient notification and underreporting must be assumed. HFRS symptoms resemble those of many other febrile infections, hence cases might often not be diagnosed specifically. Humans might be extensively exposed to this novel virus since *N. hispida* is frequently found in close

proximity to human settlements. The broad spatial distribution of *N. hispida* throughout Sub-Saharan Africa (see appendix A.1) possibly allows for a wide distribution of MGBV. However, nothing is known about the pathogenic potential of bat-borne hantaviruses. A good indicator to predict the pathogenic potential of hantavirues on humans seems to be knowledge on receptor use (Gavrilovskaya et al. 1998, 2008; Schönrich et al. 2008; Song et al. 2007). Obviously, this requires isolation of the respective virus which was not yet achieved for any bat-borne hantavirus. Despite the lack of a virus isolate, the data presented here suggest bats as further extension of the natural hantavirus host range.

6.1.2 Paramyxoviruses in three bat species

Samples obtained from two fruit bat and one insectivorous bat species in West and Central Africa were tested for the presence of paramyxovirus (PMV) RNA. Viral sequences were detected in multiple organs as well as in excreta, with an overall detection rate of 3.7% (see Weiss et al. 2012a). All animals in this study appeared clinically healthy upon sampling. Consistent with asymptomatic seroconversion of experimentally infected bats (Williamson et al. 1998; Middleton et al. 2007) it might well be that these viruses do not cause overt clinical disease in bats, despite evident infection. High detection rates in spleen suggest a distribution of virus in lymphoid organs, as known for other PMVs, for example *Nipah virus* (NiV) that has been shown to efficiently disseminate within a host by hijacking lymphocytes (Mathieu et al. 2011). High viral loads were found in urine. The detection of viral sequences in only one faecal sample might be due to contamination of the sample with urine. It is known for henipaviruses that they circulate in the genitourinary tract of bats (Williamson et al. 1998; Middleton et al. 2007), which provides an efficient way of virus transmission. In Bangladesh, humans became infected after consuming date palm sap contaminated with bat urine and saliva (Luby et al. 2006) and bat excreta are the suspected cause of *Hendra virus* (HeV) transmission between bats and horses (Williamson et al. 1998). Infection of domestic pigs in Ghana (Hayman et al. 2011) might also be a result of contact with bat excreta, which is especially troubling because pigs have acted as amplifying hosts in previous NiV outbreaks in humans (Field et al. 2001).

More than 27% of all *Eidolon helvum* tested during this study were shown to harbour PMV sequences. Moreover, these are 33% of all bats that were destined for bushmeat markets. This is alarming for several reasons: First, *E. helvum* are very large fruit bats and are extensively hunted for bushmeat (Mickleburgh et al. 2009; Kamins et al. 2011). Animals are handled without any protection by hunters, opening possibilities for interspecies-transmission through handling, preparation, and consumption. Second, humans are additionally exposed to these viruses through environmental contamination with bat excretions and saliva, a risk that is elevated by the habit of *E. helvum* to roost in the middle of cities. Third, bats of the genus *Eidolon* are closer related to *Pteropus* sp. than to any of the bat species occurring in mainland Africa (Almeida et al. 2011). If henipaviruses co-evolved with their pteropodid hosts, as data on NiV outbreaks suggests (Wild 2009), *Eidolon* bats are the most likely candidates to harbour viruses with similar properties. Indeed, African Eidolon Paramyxoviruses (EPMV) with close relationship to HeV and NiV were shown to carry a specific catalytic motif (GDNE) in their polymerase gene (Drexler et al. 2012),

which has so far only been detected in Autralian and Asian bat paramyxoviruses, while other members of the Mononegavirales carry a GDNQ motif instead (Wang et al. 2001; Miller 2003; Magoffin et al. 2007; Sasaki et al. 2012).

The origin of paramyxoviruses When bat-derived PMV sequences are compared to other PMVs they form separate groups in a phylogenetic tree. In the subfamily *Paramyxovirinae* EPMVs cluster in three distinct groups, regardless of their spatial origin (West or Central Africa), suggesting virus exchange between colonies during annual migration. Viruses belonging to the genus *Henipavirus* group within the diversity of these novel paramyxoviruses. Another recently described and well characterised virus, *Cedar virus*, was isolated from an Australian fruit bat and classified as belonging to *Henipavirus* (Marsh et al. 2012) also groups therein, yet further distant from HeV and NiV than a group of EPMVs. The single *Myonycteris*-derived sequence that groups with EPMVs might be a result of an interspecies-transmission event, since *Eidolon* and *Myonycteris* bats live sympatric in Gabon and elsewere. The close association of many fruit bat-derived sequences with the genus *Henipavirus* is not surprising, especially not for EPMVs with respect to evolutionary relationships between *Eidolon* and pteropodid bat species. All sequences found in *Myonycteris* species originate from Central Africa, however only few organ samples from West African animals were tested. It was recently shown that Central and West African little collared fruit bats do not constitute a panmictic unit but rather two genetically isolated species, *My. torquata* in Central Africa and *My. leptodon* in West Africa (Nesi et al. 2012). It would be of interest to sample more *My. leptodon* individuals so as to determine whether a possible correlation exist between the absence of gene flow between West and Central African little collared fruit bats and the extinction (or absence of spread) of PMVs from one stock to the other.
The great variety of bat-derived PMVs as compared to those described in other species might lead to the suggestion that bats could be at the origin of the emergence of many PMVs currently circulating in mammals belonging to other orders. However, parsimony based host switch reconstruction reveals primates as the main donors of presently circulating PMVs, while bats only serve as main donor for rodent PMVs. Two things have to be kept in mind: First, the high detection rate in bats reflects sampling effort, which, to our knowledge, was not conducted on any other mammalian species. Second, all bat viruses have been detected in clinically healthy individuals, while other PMVs originate mostly from sick or deceased individuals. As a consequence, we know nothing about the variety of PMVs in other healthy mammals. Even more surprisingly, and despite including more bat-derived sequences in the present analyses, our finding is in contrast to a previous study claiming bats as the main donors of PMVs (Drexler et al. 2012). Our result was confirmed when data were re-analysed using a probabilistic approach (likelihood ancestral state reconstruction), which accounts for branch length rather than tree topology only (Pagel 1999). However, analyses are based on a relatively short fragment (413 bp) and overall number of host switches reflected by the phylogenetic trees is very low. This might introduce some biases and makes the dataset particularly sensitive for mislabelling. More data, espcially on the variety of PMVs in healthy primates and other mammals, will be needed before the question of paramyxovirus origin can be answered reliably.

6.1.3 A novel reovirus related to human pathogenic viruses

A novel reovirus has been isolated from pooled blood of three individuals of the Duke Of Abruzzi's Free-tailed Bat (*Chaerephon aloysiisabaudiae*) captured in the Taï Forest National Park in Côte d'Ivoire. The virus was tentatively named *Taï Forest Reovirus* (TFRV) and its sequence identifies it as a relative of the members of the genus *Coltivirus*. Thus, it represents the first reovirus isolated from bats outside the genus *Orthoreovirus* and the first bat-borne reovirus in Africa. The isolation of TFRV from blood is in line with its close relationship to coltiviruses. *Colorado tick fever virus* (CTFV), the type species of coltiviruses, replicates in erythropoietic cells and produced virus is maintained in red blood cells (Emmons 1988).
The only biological function that can be assigned to a coding region with some certainty is for segment 1 of TFRV, which probably codes for the RNA-dependent RNA polymerase (RdRp). It is the only sequence for which homology to known sequences can be postulated safely and it harbours two functional motifs that are common in RdRps of other reoviruses (Attoui et al. 2002). Maximum amino acid sequence identity levels between coding sequences of allocated TFRV segments and coltivirus sequences range between 28 and 39%. These values might be even lower for the remaining segments, which makes it difficult to map yet 'orphan' sequence reads (sequences obtained from Sanger or next generation sequencing that do not show any identity to sequences found in GenBank). Specific 'blind' PCRs with 'out-primers' (see section 4.6.6) trying to connect orphan reads could provide longer sequence stretches that might allow for identification of further relevant reads. Another approach for sequence completion and allocation would be to separate genomic segments in a gel and extract bands to use them in random amplification or amplification with primers targeting the conserved ends of the segments.
The induction of a cytopathic effect on a wide range of human cells in vitro could be a first hint in favour of the ability of TFRV to infect humans. CTFV causes a febrile disease, Colorado tick fever (CTF), in humans. These symptoms are common in tropical regions and can account for a number of different diseases. In Africa, especially in remote areas where *Ch. aloysiisabaudiae* is found, functioning health care systems are rare and most of the time no diagnostic test is performed. Hence, if TFRV would cause a disease similar to CTF in humans, it will most likely remain unidentified.

Based on present data we cannot infer whether *Ch. aloysiisabaudiae* is an accidental host or represents the true reservoir of TFRV. No virus was detected in any other bat species but in all three tested *Ch. aloysiisabaudiae* individuals. This might argue against those bats being accidental hosts but rather for a host restriction of the virus. However, ixodid ticks, which serve as vector for CTFV and the European *Eyach virus* (EV), are present in Africa and could provide the possibility for spread of the virus. Similarly, *Ch. aloysiisabaudiae* is widely distributed. Besides the forest zones in Côte d'Ivoire and western Ghana, it is also present in Central Africa along a stretch from Cameroon to South Sudan and Uganda. Since populations are spatially separated it would be of interest to look for TFRV in central African individuals. With respect to the pathogenic potential of other colitiviruses it would be of value to establish a serological test

to screen humans from the Taï region for antibodies against TFRV. Also ticks and other animals could be captured and tested for virus or antibodies to explore the epidemiology and potential impact of this novel reovirus in the region of the Taï National Park and along the distribution of ixodid ticks and *Ch. aloysiisabaudiae*.

6.1.4 Filoviruses in African bats

To investigate the broader distribution of filoviruses in Africa, 773 bat blood samples originating from Sierra Leone (2009), Côte d'Ivoire (2006) and the Republic of Congo (2009 and 2010) were screened for antibodies against *Ebola-* (EBOV) and *Marburg* (MARV) *virus*. In total, 1.3 and 1.9% of all samples tested positive for EBOV and MARV antigen, respectively, but values differed widely depending on bat and virus species.
In the test applied in this study, sera reacted with the nucleoprotein (NP) or with viral proteins VP30/ 35/ 40 (MARV) or VP40 (EBOV) in Western blot. These proteins are predominantly found on protein gels stained with Coomassie blue (Kiley et al. 1988). No blood sample reacted with the glycoprotein (GP), an observation in line with previous studies on humans with asymptomatic EBOV infection (Leroy et al. 2000), and most likely due to the high degree of glycosylation of GP. Three individuals (two *Hypsignathus monstrous* and one *Rousettus aegyptiacus*) tested positive for both, EBOV and MARV. Since both viruses only show low antigenic relation we can conclude detection of dual infection in three individuals. This is also supported by the fact that one serum sample from *H. monstrosus* was reactive against different proteins of both viruses. Nevertheless, cross-reactivity between EBOV and MARV as well as with other viruses cannot be completely excluded. Immunofluorescence and blocking assays with recombinant proteins could be done to further confirm specificity. However, to our knowledge no antigenic relationship has ever been reported to any of the most closely related viruses, namely paramyxo- and rhabdoviruses.

Marburgvirus In previous studies, MARV-specific antibodies (MARV-Abs) were most frequently detected in the cave-dwelling fruit bat *R. aegyptiacus* (detection rate 2.4–21.5%, average 11.9%) as compared to other bat species (0.3–1%), and also active infection has been shown in these animals (Swanepoel et al. 2007; Towner et al. 2007). Consequently, most following studies focused on this bat species (Towner et al. 2009; Kuzmin et al. 2010). Reports about antibodies or viral RNA found in other bat species have been restricted to single individuals, except one insectivorous species, *Rhinolophus eloquens* (9.7% antibody positive) (Swanepoel et al. 2007; Pourrut et al. 2009). It is noteworthy, that Rhinolophinae (Rhinolophidae) are closer related to megabats than to any other microchiropteran species (Almeida et al. 2011).
The present study confirms the presence of MARV-Abs in two bat genera (*Hypsignathus* and *Rousettus*), and shows antibodies in two additional bat species: *Myonycteris torquata* and *Mops thersites*. Even though absolute numbers are low and thus values have to be taken with care, results are remarkable for *H. monstrous* and *My. torquata*. We confirmed more than 8% of all *H. monstrosus* samples positive for MARV-Abs by immunoblotting, whereas previous studies only report 1% positive in ELISA assays (Pourrut et al. 2009). Despite testing of almost 1,000

individuals nothing has so far been reported connecting *My. torquata*a to MARV in Central Africa (Pourrut et al. 2009). Myonycterini were the most abundant fruit bats sampled in our study (307 individuals); more than half (n = 183) of them captured in West Africa where no broad serological studies have yet been conducted. Here, six out of seven samples that were confirmed in Western blot originated from West Africa. Whether this is a result of sampling bias or whether phylogeographic differences in Myonycterini (Nesi et al. 2012) play a role remains subject for future studies. Both abovementioned bat species are rather living solitary and only meet for mating, a behavior that should limit virus spread and persistence in a given population, yet seems to be sufficient to maintain virus. In contrast, *Mops* sp. show similar group roosting behavior like *Rousettus* bats, but are frequently found in close proximity to humans like in attics. If bats of this genus were confirmed as transmitters of filoviruses, it would raise new concern about the possibility of future transmission events.
Predictions modeling the geographic distribution of MARV point towards dry areas in the eastern and south-central parts of the continent as the main risk zones and, interestingly, the outbreak of MARV in Uige (Angola) in 2005 occurred in such a previously predicted risk zone (Peterson et al. 2006). We could confirm MARV antibodies in bats from Central and West Africa, demonstrating that MARV has a wider spatial distribution and a broader host range among different bat species than initially suggested (Peterson et al. 2004, 2006). To our knowledge, no major outbreaks of MARV VHF have been reported from West Africa, specifically from the exact origin of the samples in Côte d'Ivoire where a long-term project is in place investigating causes of mortalities in every wild primate found dead since 1996 (Leendertz et al. 2006). Despite the absence of data on outbreaks in these regions it cannot be excluded that individual cases or smaller outbreaks have gone unnoticed. Given the predominant occurrence of human MARV-related disease outbreaks in East Africa and the broad distribution of MARV also in other tropical rainforest zones, it will be important to investigate determinants associated with virus transmission and consecutive outbreaks to pinpoint risk factors for MARV-related disease outbreaks in humans.

Ebolavirus In contrast to MARV, EBOV was predicted essentially in areas surrounding known occurrence points in Africa, coinciding with evergreen broadleaf forest distribution (Peterson et al. 2004). This is consistent with the geographic distribution of the three fruit bat species that are currently the most likely candidates for being EBOV reservoirs: *Myonycteris torquata*, *Epomops franqueti*, and *Hypsignathus monstrosus*. In these species, captured in Gabon and the Republic of Congo (Central Africa), EBOV-specific antibodies (EBOV-Abs) have previously been detected in 4–5% of all tested individuals (Leroy et al. 2005; Pourrut et al. 2007, 2009). A recent study from Ghana (West Africa) detects 36.4% (32/ 88) samples reactive with EBOV antigen in ELISA. However, this is the only study using NP proteins (ZEBOV and RESTV) instead of full virus protein, and only seven (8%) of the ELISA positive samples were confirmed with Western blot (Hayman et al. 2012).
In the present study, the overall detection rate of 3.5% in the abovementioned species is comparable with results from previous studies; however, values differ widely between species. Despite comparable sample numbers, EBOV-specific antibodies were more frequently (*H. monstrosus*) or

exclusively (*Epomops* sp.) detected in bats from West Africa. Interestingly, these are the same bat species as previously reported with relatively high positivity rates (12.5 and 11.1% confirmed by immunoblot) in West Africa (Hayman et al. 2012). The detection of EBOV-specific antibodies in *Myonycteris* bats from West Africa is again especially interesting with respect to recent phylogeographic data about Myonycterini (Nesi et al. 2012): Nesi and colleagues conclude that the high genetic distance observed between *Taï Forest ebolavirus* and *Zaire ebolavirus* (ZEBOV) can be explained by the genetic distance of their respective reservoir species, *My. leptodon* and *My. torquata*. Blood samples in the present study were tested exclusively against ZEBOV antigen, but studies on human IgG show cross-reactivity between all five EBOV species (MacNeil & Rollin 2012). Quantitative blocking assays might help to solve this question.

The ecology of filoviruses In the present study, we did not detect active infection in any bat. Based on serological data only it is not possible to draw firm conclusions on the wave-like spread of ZEBOV through Central Africa, as suggested by Walsh et al. (2005).
In 2011 the first European filovirus was reported in deceased Schreiber's long-fingered bats (*Miniopterus schreibersii*) in a cave in spain. *Lloviu virus* is suspected to be pathogenic for these animals (Negredo et al. 2011) and would therefore represent the only filovirus harming bats. Before that, *Reston ebolavirus* (RESTV) has long been the only filovirus not endemic to Africa and represents the only EBOV variant that, despite exposure, does not cause disease in humans (reviewed in Miranda & Miranda 2011). In 2011 there was a first report linking RESTV to bats when antibodies were detected in the common Rousette (*Rousettus amplexicaudatus*). Animals were captured in the Philippines, the probable origin of RESTV. Interestingly, no antibodies were detected in bats from the other 16 bat species tested (Taniguchi et al. 2011). *R. amplexicaudatus* is related to the Egyptian Rousette (*R. aegyptiacus*), the putative primary reservoir host of MARV. The same species is also present in Indonesia, were EBOV-specific antibodies have recently been described in Orangutans (Nidom et al. 2012), further extending the geographic and host range of EBOV. This was recently affirmed by the detection of ZEBOV-Abs in *Rousettus leschenaultii* in Bangladesh (Olival et al. 2013).
A major concern about the spread of EBOV was raised in 2008 when swine have been proven as hosts for RESTV and multiple variants of the virus have been isolated from deceased animals (Barrette et al. 2009). Antibodies against RESTV have been detected in pig farmers and it was shown that pigs are able to amplify and transmit EBOV efficiently, even without direct contact (Barrette et al. 2009; Kobinger et al. 2011; Weingartl et al. 2012). A similar scenario has been responsible for the disease outbreak leading to the emergence of *Nipah virus*, a paramyxovirus belonging to the same order *Mononegavirales* like filoviruses (Johara et al. 2001).

Even though accumulating evidence point towards bats as primary reservoir for filoviruses (Leroy et al. 2005; Biek et al. 2006), there is still some uncertainty, at least for EBOV. Seroprevalence rates detected in bats are generally below 10%, but one would expect much higher rates in the virus' reservoir. Also, no live EBOV has yet been isolated from bats. Summarising all these information it becomes clear that we are far from fully understanding the ecology of filoviruses

and much more effort has to be done before we might be able to prevent disease outbreaks caused by filoviruses.

6.2 The role of bats as hosts for emerging viruses and its impact on human health

The present study confirms the importance of bats as hosts for emerging viruses, and luckily novel viruses described here have not yet had devastating impact on humans.
Successful virus spill-over between different species is usually a result of repeated transmission events and depends on the viral richness found in natural reservoir species (Wolfe et al. 2005; Turmelle et al. 2011). Having this in mind the detection of a vast variety of novel paramyxoviruses (PMVs) in different bat species raises particular concern, especially since the viruses detected in bat bushmeat samples are those most closely related to highly pathogenic Hendra and Nipah viruses. It needs to be mentioned, however, that reported viral richness in a given species is always correlated with improved detection methods and sampling effort. Both have been intensified, especially for PMVs in bats, in the recent past (Hayman et al. 2011; Drexler et al. 2011, 2012; Kurth et al. 2012; Weiss et al. 2012a; Marsh et al. 2012; Baker et al. 2012; Wilkinson et al. 2012; Sasaki et al. 2012; Barr et al. 2012). Despite absent surveillance systems we can assume that increasing disease or mortality among bat hunters would not have gone unnoticed, at least in our research areas. The lack of such reports hence indicates either low or no transmission, or transmission of variants not pathogenic for humans.
Similar conclusions can be drawn from results on the seroprevalence of *Ebola-* and *Marburgvirus*. Despite an extended host and geographic range of filoviruses reported here transmission to other mammals seems to be rare. While our data indicate higher seroprevalences in western than in central African bats, human filovirus outbreaks are much more common in Central Africa. Reasons for that might be connected to human comportment, for example different poaching behaviour, or yet unidentified ecological features. Most past human outbreaks could be traced back to the handling of primate bushmeat while primates acquire the infection probably by sharing fruit sources with bats (Leroy et al. 2004; Formenty et al. 1999).

The potential of bats as hosts for emerging viruses is also confirmed by the detection of a novel hantavirus and the isolation of a coltivirus-related reovirus (Weiss et al. 2012b). Both virus species have not been associated with bats before and there are no reports about either virus being transmitted to humans. Yet since symptoms possibly resemble those of many other (more common) diseases single events might have gone unnoticed. Interestingly, both viruses have segmented RNA genomes allowing for reassortment, which indeed has already been observed for both virus families (Chu et al. 2011; Maan et al. 2012; Guo et al. 2013). Coltiviruses have previously been restricted to North America and Europe. Isolation of *Taï Forest reovirus* from an African bat extends the host and geographic range of coltiviruses and confirms tropical Africa as hotspot for emerging virus families. Successful infection of human cell lines raises concerns about the transmissibility of this virus to humans. In contrast, hantaviruses are known to circulate in West

Africa as they have been detected in other small mammals in the last years (Klempa et al. 2010). The host range extension revealed during the present study underlines how little we know about the ecology of these viruses in Africa and supports the need for future research.

6.3 Anthropogenic influence on the emergence of novel viruses

It is evident that human intrusion in pristine ecosystems has major impacts on the emergence of viral disease (Daszak et al. 2001). Intensified contact between wildlife and humans through logging, agricultural use of deforested areas, and poaching raises possibilities for successful pathogen spill-over. Thereby, hunting, trade, and consumption of bushmeat probably have the most direct influence. Handling routines of bushmeat seem to differ regionally: It has been reported that in Ghana, West Africa, bats are sold dead and smoked (Kamins et al. 2011), whereas in Brazzaville, Central Africa, animals are sold alive (personal communication with local hunters). Hunting habits also contribute indirectly to the risk of disease emergence by influencing the threatening status of specific species. Indeed, hunting is suspected to be the main factor that led to the IUCN near-threatened status of *Eidolon helvum* (Mickleburgh et al. 2008d), and many flying fox species in Asia and Madagascar are close to extinction due to hunting (Mickleburgh et al. 2009; Jenkins & Racey 2008). The loss of biodiversity itself has been proven to be a factor triggering disease emergence (Keesing et al. 2010) and obviously, loss or decimation of one species has a broader ecological impact. Functioning ecosystems rely on bats as seed dispersers and to control insect populations, which also play a major role as vectors in pathogen spread. A study on arthropod-transmitted viruses in and around the Taï Forest National Park could show that disturbed habitats support proliferation of endemic viruses more efficiently than pristine primary rainforests (Junglen et al. 2009). Whether or not spill-over events lead to sustained transmission of a given pathogen is probably also influenced by the immunological status of the local population since underlying chronic infections (HIV, parasitic infections, etc.) might weaken the immune system and consequently facilitate pathogen spread. Eventually, logging and ecotourism increase contact between wildlife habitats and the developed world, thereby allowing pathogens to escape more easily from remote geographic areas. Hereby, especially ecotourism represents a dilemma since several studies have shown its positive effects on conservation aspects (Krüger 2005; Köndgen et al. 2008).

6.4 General discussion

Emerging diseases originating from bats have had devastating effects on wildlife populations (e.g. *Ebolavirus* decimating African great ape populations), livestock (e.g. culling of pigs as a result of the occurrence of *Nipah virus* in Malaysia), and humans (e.g. SARS-epidemic, recurrent *Nipah virus*-caused disease outbreaks in Bangladesh). However, sucessful cross-species transmission remains a complex process involving multiple restrictions: Environmental and demographic aspects

(e.g. contact rates between donor and recipients, density of recipient host population, intermediate and amplifying hosts) are largely influenced and driven by anthropogenic interferences. In contrast, host/ species barriers (e.g. cross-immunity, cellular receptors, innate immunity) and existing pathogen host range (determined by genome type [RNA vs. DNA, ds vs. ss] influencing the ability for recombination and reassortation, adaptation, mode of transmission) are non-influencable pathogen traits.

Since it is currently impossible to foresee emerging virus transmission events we rely on rapid and specific diagnostic to prevent devastating consequences. This goal is impaired by the fact that diseases associated with virus families detected in bats display a wide range of unspecific symptoms: Fever, headache, or nausea are common in tropical regions and underlying etiologic agents are rarely diagnosed, especially when diseases are not fatal and do not lead to broad epidemics. Specific diagnostics are time and money consuming and require educated personnel and equipment, all of which is rare or absent in remote African rainforest areas. Both, specific and broad diagnostic tests are needed to gain insight into the ecology of emerging pathogens. Prevalence studies on wildlife reservoirs will help to identify the ecology of certain suspected emerging pathogens and their hosts, knowledge that is urgently needed to prevent future transmission of bat-borne pathogens to humans. By generating genetic information on fast evolving viruses we might also gain information about host evolution (Biek 2006). However, if we want to understand 'true' transmission chains, systematic sampling is needed, ideally on the interface where contact between wildlife and humans is intensive, such as bushmeat markets. Besides sampling of animals this should also include humans, predominantly hunters, vendors, and consumers of bushmeat. Serological screenings of humans could give first hints to identify pathogens that have already crossed the interspecies barrier, despite overt disease.

Remoteness is what currently mainly protects the developed world from novel viruses emerging locally in tropical rainforests, but this 'protective barrier' is continuously decreased by human encroachment. Despite sensitive use of natural resources, road constructions that come along with logging activities or ecotourism ease access to previously pristine rainforest areas and thus pose a serious threat to local fauna. While human behaviour certainly influences pathogen dynamics and transmission risk in a given population, there are also some influential factors not directly linked to human intrusion, like seasonality and migration of host species. Seasonality linked to climate or pregnancy and lactation was proven a risk factor for a number of viral diseases in bats (McFarlane et al. 2011; Pourrut et al. 2007; Plowright et al. 2008; Turmelle et al. 2011; Drexler et al. 2011), and shown to have an impact on transmission risk to humans (Amman et al. 2012). The influence of migration has recently been reviewed by Altizer et al. (2011): While migration is commonly associated with a high potential to spread viruses and facilitated cross-species transmission recent findings assign that it can also have opposite effects: By allowing hosts to escape from infected habitats and reducing disease levels when infected animals do not migrate successfully, it may lead to the evolution of less-virulent pathogens in a given population (Altizer et al. 2011).

7 Outlook

The importance of bats as hosts for emerging viruses has been increasingly appreciated during the past 10–15 years and the present study confirms how little we actually know about the 'zoonotic pool' harboured by African bats. With epidemics like SARS it has become obvious in recent years, that emerging viruses are no longer a problem of the so-called 'third world'. While it is questionable whether we will ever be able to foresee successful pathogen inter-species transmission, we need to do whatever is possible to prevent or at least attenuate the devastating effects on wildlife, humans, and economy. The present study contributes to this aim by adding to the growing body of knowledge currently generated on emerging viruses in African bats. We need to understand the ecology of those pathogens and the factors triggering disease outbreaks to eventually establish suitable prevention measures. These should include training of medical and veterinarian staff and providing suitable equipment. However, for sustaining success changes need to occur where diseases emerge, hence in remote African regions. Any prevention measures rely on acceptance of the local population, so obviously communication strategies are a key factor. By also including local authorities we need to educate local populations and make them more vigilant to achieve behavioural changes in agricultural practices and bushmeat handling. A good example is the management of Lassa fever in Nigeria. There, referential clinics have been introduced to reduce the burden of Lassa fever, but have proven to act as clinic for patients with undiagnosed febrile illnesses, thereby becoming sentinel sites for emerging infectious diseases (Gire et al. 2012). Future research must aim in international and interdisciplinary approaches including ecologists, virologists, veterinarians, health professionals, social and environmental scientists, and also politicians, to understand the ecology of emerging pathogens and to minimize the impact of bat-borne zoonotic diseases.

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Appendix

A.1 Bats

The following section summarises some basic information on the ecology of those bat species relevant for the present study.

A.1.1 Insectivorous bat species

Nycteris hispida The hairy slit-faced bat *Nycteris hispida* (Nycteridae) is an insectivorous bat species in the superfamily Rhinolophoidea and occurs in sub-Saharan Africa. It is one of the most widely distributed slit-faced bats, ranging from closed rainforests to dry steppe (Mickleburgh et al. 2008a). Nycterids forage mainly by "gleaning", that is picking their prey from the ground or other surfaces. *N. hispida* is a synanthropic species frequently found roosting in disused huts and granaries, which brings them in contact with other commensal mammals and humans.

***Hipposideros* sp.** The genus Hipposideros (Hipposideridae), commonly known as roundleaf bats, is one of the most diverse bat genera and includes more than 70 species (Wilson & Reeder 2005). These bats use caves, man-made underground chambers, buildings, and hollow trees as retreats. Some species roost singly while others form colonies with only few or up to several hundred thousand individuals and are frequently found associated with other bats in their day roosts. Hipposideros bats fly lower than many other bat species and catch insects like moths, beetles, termites, and cockroaches while flying (Nowak 1994).

Chaerephon aloysiisabaudiae The Duke of Abruzzi's free-tailed bat *Chaerephon aloysiisabaudiae* (synonym: *Tadarida aloysiisabaudiae*, [Molossidae]) occurs in tropical dry and moist lowland forests as well as in moist savannahs in Côte d'Ivoire and Ghana, and in Central Africa along a stretch at the borders between Cameroon and Gabon, to South Sudan, and Uganda. The species feeds on insects and is suspected to roost in hollow trees (Mickleburgh et al. 2008c).

***Mops* sp.** The genus Mops (Molossidae) contains twelve species, which are endemic to sub-Saharan Africa and feed on flying insects. They commonly roost in group sizes of several dozen to hundreds of individuals, which are found in a variety of roost types, often in proximity to humans, like hollow trees or attics (Kingdon 1997).

A.1.2 Pteropodidae

Eidolon helvum The straw-coloured fruit bat is the second largest fruit bat in Africa and broadly distributed across the lowland rainforest and savanna zones. The genus is special among African bats for several reasons: Eidolon forms a separate lineage within the Pteropodidae and is more closely related to Asian and Australian pteropodid genera than to the other African fruit bat species (Almeida et al. 2011). Eidolon populations migrate several thousand kilometres from the West African forest into savanna zones during the wet season following fruit availability (Thomas 1983; Richter & Cumming 2006). They form large colonies with thousands up to several million individuals, which disperse into smaller groups during travel. During residential phases they are frequently found in urban areas. *E. helvum* is the bat species most heavily harvested for bushmeat in West and Central Africa, and hunting is believed to be a major factor in reported population declines (Mickleburgh et al. 2008d).

Myonycterini The tribe Myonycterini comprises seven fruit bat species, all of which are endemic in Africa: *Myonycteris torquata*, *My. leptodon*, *My. brachycephala*, *My. relicta*, *My. angolensis*, *Megaloglossus woermanni*, and *Me. azagnyi*. *My. brachycephala* and *My. relicta* are restricted to the island of São Tomé and East Africa, respectively. *My. torquata* and *My. leptodon* are distributed across the Afrotropical forest zone but separated by the Dahomey Gap, a savannah corridor in Ghana, Togo and Benin separating the Upper Guinea Forest in West Africa from the large Congo Basin Forest in Central Africa (Salzmann & Hoelzmann 2005). *My. torquata* is found in Central Africa while *My. leptodon* occurs in West Africa (Nesi et al. 2012). Similarly, two geographic clades exist for Megaloglossus: *Me. azagnyi* (West Africa) and *Me. woermanni* (Central Africa) (Nesi et al. 2012). All species are generally associated with tropical lowland forest but may also be encountered in forest-grassland mosaics. Myonycteris is known to roost singly or in small groups and subadult *My. leptodon* males appear to be migratory (Thomas 1983). In contrast, little is known about roosting behaviour of Megaloglossus, but they do also not seem to roost in groups. While the latter represent the only obligate nectarivorous bat species in Africa, Myonycteris is feeding on both fruits and flowers (nectar and pollen) (Weber et al. 2009).

Rousettus aegyptiacus The Egyptian rousettes are fruit bats found from arid to moist tropical and subtropical biomes. They are roosting exclusively in humid caves, which can be natural caverns or artificial/ antrophogenic structures. *R. aegyptiacus* build colonies with hundreds or up to several hundred thousand individuals and often roost with other bat families like Hipposideridae. They feed on soft fruits, flowers, and leaves, and often forage in orchards. This genus is one of the Old World fruit bat using echolocation during flight (Kwiecinski & Griffiths 1999). *R. aegyptiacus* is not recognised as migratory bat species but one individual dispersed over a distance of 500 km (Amman et al. 2012).

Hypsignathus monstrosus The hammer-headed fruit bats are widely distributed across West and Central Africa, ranging from Sierra Leone to western Uganda, and live in lowland tropical

forests, riverine forests, swamps, and mangroves. They roost in trees, either in small groups or singly, and mainly feed on soft fruit (Langevin & Barclay 1990).

Epomops buettikoferi Büttikofer's epauletted fruit bats mainly occur in the Upper Guinea forest block in West Africa and are typically found singly or in small groups. This species is not known to migrate (Mickleburgh et al. 2008b).

A.2 Study locations

This section provides more detailed information on climate, vegetation, fauna, and known anthropogenic impact of the locations where bats were sampled for the present study.

A.2.1 GOLA: *Trans-boundary Peace Park* (Sierra Leone, Liberia)

The *Trans-boundary Peace Park* unites the Gola Forest Reserve in Sierra Leone (75,000 ha) and the Lofa and Foya Forest Reserves in Liberia (80,000 ha and 100,000 ha respectively) (Africa and Europe in Partnership 2013). The fauna of the park includes 50 mammal species. The forest is part of the Upper Guinea Forest that belongs to the global 25 biological diversity hotspots, which best represent the world's diversity (Gola Rainforest National Park 2013; Conservation International 2013) There is a marked dry season from December to March. Mean annual precipitation of the area is around 2500–3000 mm with July and August being the wettest months. Predominant vegetation types are moist evergreen and moist semi-deciduous forest. Large parts of Gola North in Sierra Leone are assumed to have never experienced anthropogenic influences such as logging or farming, while some other areas are lacking large trees due to selective logging. Parts of the national park are still affected by illegal human activities such as bushmeat hunting and diamond mining (Weber & Fahr 2011).
Bats in this area were sampled on ten study sites in Sierra Leone between 19th February and 11th April 2009. A second sampling period included animals from three study sites in Liberia and two study sites in Sierra Leone between 8th February and 11th March 2011.

A.2.2 PUTU: *Putu Range* (Liberia)

The *Putu Range* lies in a mining concession covering 42,500 ha and is situated in the southeast of Liberia. It consists of two prominent mountain ridges (Mt. Jideh + Mt. Montroh and Mt. Ghi) roughly running from northeast to southwest and varying in habitat conditions. The ridges and slopes of Mt. Jideh and Mt. Montroh are covered by mature forest, but exploration roads and drilling pads have already had a major impact on the integrity of forest habitats. In contrast, Mt. Ghi shows limited signs of past logging activities, and forest cover appears to be rather undisturbed. High hunting pressure is present throughout the concession area (Weber et al. 2011).
Animals in this area were sampled on eight sites between 3rd November and 2nd December 2010.

A.2.3 PNT: *Parc National de Taï* (Côte d'Ivoire)

The Taï National Park lies in the southwest of Côte d'Ivoire, on the border to Liberia. With an area of 536,300 ha it is the largest protected rainforest area in West Africa since its creation in 1972. It is mainly mature tropical lowland forest with patches of deciduous trees on hill tops and the climate is characterised by two dry seasons (major, November- February; minor, July-August) and two wet seasons (major, August-October; minor, March-June). Annual rainfall summarises to 1800 mm and average temperature is 24-28°C (N'Goran 2012, Fahr 2011). The fauna of the park contains 47 of the 54 species of large mammal known to occur in Guinean rainforest including five threatened species (UNESCO 2013a). This unique fauna is threatened by increasing human density and the increase of poaching that goes along with that (N'Goran et al. 2012).
Bats were captured on eleven sites in vicinity of a research station in the southern part of the park between 8th November and 5th December 2006.

A.2.4 PNOK: *Parc National d'Odzala-Kokoua* (Republic of Congo)

The *Parc National d'Odzala-Kokoua* (PNOK) in the Republic of Congo is situated on the north-western part of the country, on the boarder to Gabon. It was created in 1935 with an area of 285,000 ha, extended to 1,354,600 ha in 2001 (African Parks 2013a) and is declared a UNESCO Man and Biosphere Reserve since 1977 (UNESCO 2013b). Vegetation type is mainly primary forest, interspersed with savannah regions in the southern part, which also harbours an abandoned research facility and a camp for ecotourism (personal observation). The fauna of the park includes 114 mammal species, including chimpanzees and gorillas, which give the park its reputation (African Parks 2013b). Hunting pressure, especially in the inner parts of the park seems to be rather low (personal observation).
Bats were captured on five spots in the park along the Mambili river and on two adjacent villages between 12th August and 21st September 2009.

A.2.5 IFO: *Industrie Forestière d'Ouesso* (Republic of Congo)

The *Industrie Forestière d'Ouesso* (IFO) is a subsidiary of the Danzer Group that runs a logging concession of 1,160,000 ha in the north of the Republic of Congo, just south of Ouesso. Danzer committed to sustainable forest management and was certified by the Forest Stewardship Council® (FSC) in February 2009 (certificate code SGS-FM/COC-005921) (Forest Stewardship Council 2013). Selective logging leads to vegetation representing a mosaic of logged and pristine primary forest. Despite sensitive use of natural resources, the road constructions ease access to previously pristine rainforest areas thus pose a serious threat to local fauna.
Bats were captured on nine study sites between 19th February and 2nd April 2010.

A.3 Bat samples tested for hantavirus

Table A.1: Bat samples tested for hantavirus. GolaF, *Gola National Forest*, Liberia; GolaP, *Gola National Park*, Sierra Leone; IFO, *Industrie Forestière d'Ouesso*, Republic of Congo; PNOK, *Parc National d'Odzala-Kokoua*, Republic of Congo; Brazzaville, Republic of Congo; Putu, *Putu Range*, Liberia; Sabodala, Senegal

genus	type and number of samples										site	total
	blood	throat swabs	lung	liver	kidney	spleen	small intestines	pooled organs	urine	faeces		
Casinycteris sp.	4							2			PNOK, 2009	**6**
Chaerephon sp.			2								Sabodala, 2009	**2**
				1							IFO, 2010	**1**
Eidolon sp.	17							21			Brazzaville, 2009	**38**
Epomophorus sp.			2								Sabodala, 2009	**2**
Epomops sp.	12							2			PNOK, 2009	**14**
			1								GolaP, 2009	**1**
				1							Putu, 2010	**1**
Glauconycteris sp.			5	4							GolaP, 2009	**9**
	1							1			PNOK, 2009	**2**
				1							IFO, 2010	**1**
Hipposideros sp.			14	8							GolaP, 2009	**22**
	3							1			PNOK, 2009	**4**
			3								Sabodala, 2009	**3**
				4							IFO, 2010	**4**
				2							Putu, 2010	**2**
				3							GolaF, 2011	**3**
Hypsignathus sp.			1								GolaP, 2009	**1**
	25							16			PNOK, 2009	**41**
				1							Putu, 2010	**1**

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genus	type and number of samples										site	total
	blood	**throat swabs**	**lung**	**liver**	**kidney**	**spleen**	**small intestines**	**pooled organs**	**urine**	**faeces**		
Kerivoula sp.			2	2							GolaP, 2009	**4**
				2							IFO, 2010	**2**
Megaloglossus sp.			1	1							GolaP, 2009	**2**
	4										PNOK, 2009	**4**
				1							Putu, 2010	**1**
Micropteropus sp.	10										PNOK, 2009	**10**
			1								Sabodala, 2009	**1**
Mops sp.			2	1							GolaP, 2009	**3**
	4										PNOK, 2009	**4**
			2								Sabodala, 2009	**2**
				2							IFO, 2010	**2**
Myonycteris sp.			1								GolaP, 2009	**1**
	43							26			PNOK, 2009	**69**
				1							Putu, 2010	**1**
Myopterus sp.			1								Sabodala, 2009	**1**
				1							IFO, 2010	**1**
Myotis sp.			1								GolaP, 2009	**1**
Nanonycteris sp.			1								GolaP, 2009	**1**
				1							Putu, 2010	**1**
Neoromicia sp.			1	1							GolaP, 2009	**2**
			3								Sabodala, 2009	**3**
				2							IFO, 2010	**2**

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genus	type and number of samples										site	total
	blood	**throat swabs**	**lung**	**liver**	**kidney**	**spleen**	**small intestines**	**pooled organs**	**urine**	**faeces**		
Nycteris sp.	7	8	4	4	4	4	4		1	3	GolaP, 2009	**39**
		2									Nafégué, Mali, 2009	**2**
	1	1									PNOK, 2009	**2**
		5	2								Sabodala, 2009	**7**
		1		1							IFO, 2010	**2**
				1							GolaF, 2011	**1**
Nycticeinops sp.			2								Sabodala, 2009	**2**
Pipistrellus sp.			5	3							GolaP, 2009	**8**
				2							IFO, 2010	**2**
				1							GolaF, 2011	**1**
Rhinolophus sp.			2								GolaP, 2009	**2**
				1							IFO, 2010	**1**
				1							GolaF, 2011	**1**
Rousettus sp.			2								Sabodala, 2009	**2**
				1							Putu,2010	**1**
Scotoecus sp.			1								Sabodala, 2009	**1**
Scotonycteris sp.			1								GolaP, 2009	**1**
				1							Putu, 2010	**1**
				1							GolaF, 2011	**1**
Scotophilus sp.			2								Sabodala, 2009	**2**
Verspertillionidae sp.	1										PNOK, 2009	1
total	**132**	**17**	**65**	**57**	**4**	**4**	**4**	**69**	**1**	**3**		**356**

A.4 Phylogenetic trees of paramyxoviruses

Figures A.1 to A.4 show partial phylogenetic trees (compare to figure 5.7) showing the placement of novel paramxovirus (PMV) sequences in the diversity of *Paramyxoviridae*, based on a partial large gene sequence (461 nt) of the Henipa-, Morbilli-, Respirovirus (HRM) fragment.

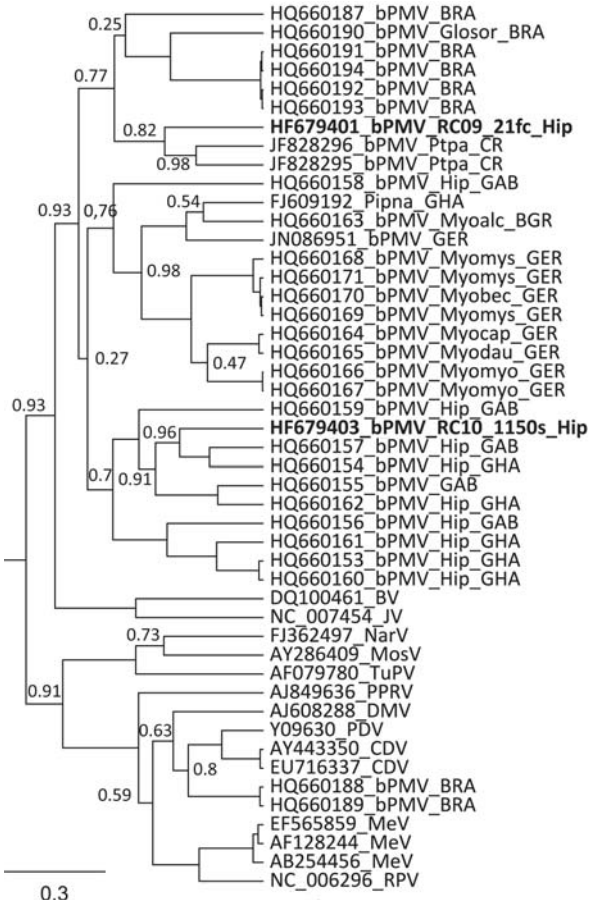


Figure A.1: Phylogenetic tree of *Paramyxoviridae* (partial): Showing the placement of novel paramyxovirus sequences from Hipposideros bats in the diversity of morbilliviruses, based on a partial large gene sequence (461 nt) of the Henipa-, Morbilli-, Respirovirus (HRM) fragment. Trees were computed by using BEAST version 1.7.1 under the assumption of a relaxed, uncorrelated lognormal clock and the Yule process speciation model. Values given are posterior probabilities (pp). For better visibility, only pp values below 1 are indicated. Scale bar indicates nucleotide substitutions per site. Paramyxovirus sequences retrieved in this study are printed in bold and named according to the following pattern: virus_ origin and year_ individual field ID and sample_ species. Virus abbreviations: bPMV, bat paramxovirus; BV, *Beilong virus*; CDV, *Canine distemper virus*; DMV, *Dolphin morbillivirus*; JV, *J-virus*; MeV, *Measles virus*; MosV, *Mossman virus*; NarV, *Nariva virus*; PDV, *Phocine distemper virus* PPRV, *Peste-des-petits-ruminants virus*; RPV, *Rinderpest virus*; TuPV, *Tupaia virus*. Country abbreviations: BGR, Bulgaria; BRA, Bazil; CR, Costa Rica; GAB, Gabon; GER, Germany; GHA, Ghana; RC, Republic of Congo. Species abbreviations: Hip, Hipposideridae; Myoalc, *Myotis alcathoe*; Myobec, *Myotis bechsteinii*; Myocap, *Myotis capaccinii*; Myodau, *Myotis daubentonii*; Myomyo, *Myotis myotis*; Myomys, *Myotis mystacinus*: Ptpa, *Pteronotus parnellii*. Sample abbreviations: s, spleen; fc, feces.

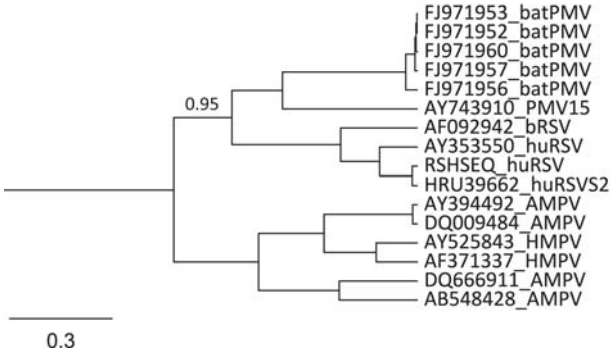


Figure A.2: Phylogenetic tree of *Paramxoviridae* (partial): Metapneumoviruses. Final tree is based on a partial large gene sequence (461 nt) of the Henipa-, Morbilli-, Respirovirus (HRM) fragment. Bayesian trees were computed by using BEAST version 1.7.1 under the assumption of a relaxed, uncorrelated lognormal clock and the Yule process speciation model. Values given are posterior probabilities (pp). For better visibility, only pp values below 1 are indicated. Scale bar indicates nucleotide substitutions per site. AMPV, *Avian metapneumovirus*; bPMV, bat paramyxovirus; bRSV, *Bovine respiratory synsytical virus*, HMPV, *Human metapneumovirus*; huRSV, *Human respiratory synsytical virus*.

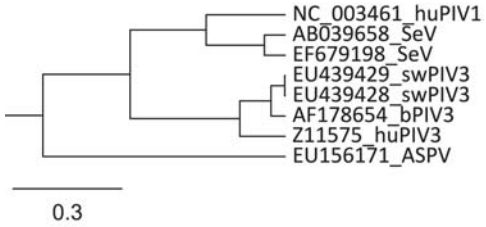
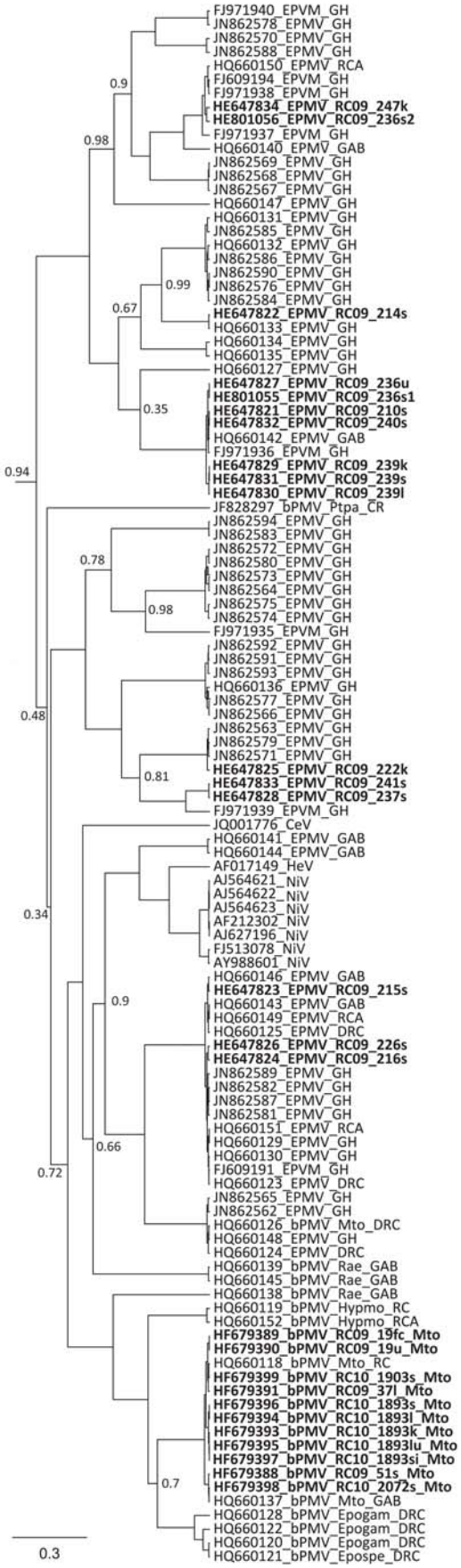


Figure A.3: Phylogenetic tree of *Paramxoviridae* (partial): Respiroviruses. Final tree is based on a partial large gene sequence (461 nt) of the Henipa-, Morbilli-, Respirovirus (HRM) fragment. Bayesian trees were computed by using BEAST version 1.7.1 under the assumption of a relaxed, uncorrelated lognormal clock and the Yule process speciation model. Values given are posterior probabilities (pp). For better visibility, only pp values below 1 are indicated. Scale bar indicates nucleotide substitutions per site. ASPV, *Atlantic salmon paramyxovirus*; bPIV, *Bovine parainfluenzavirus*; huPIV, *Human parainfluenzavirus*; SeV, *Sendai virus*; swPIV, *Swine parainfluenzavirus*.

Figure A.4 *(following page)*: Phylogenetic tree of *Paramxoviridae* (partial): Showing the placement of novel paramxovirus sequences from African fruit bats in the diversity of henipaviruses, based on a partial large gene sequence (461 nt) of the Henipa-, Morbilli-, Respirovirus (HRM) fragment. Trees were computed by using BEAST version 1.7.1 under the assumption of a relaxed, uncorrelated lognormal clock and the Yule process speciation model. Values given are posterior probabilities (pp). For better visibility, only pp values below 1 are indicated. Scale bar indicates nucleotide substitutions per site. Paramyxovirus sequences retrieved in this study are printed in bold and named according to the following pattern: virus_ origin and year_ individual field ID and sample_ species. Virus abbreviations: bPMV, bat paramyxovirus; CeV, *Cedar virus*; HeV, *Hendra virus*; EPMV, Eidolon paramyxovirus; NiV, *Nipah virus*. Country abbreviations: DRC, Democratic Republic of Congo; GAB, Gabon; GH, Ghana; RC, Republic of Congo; RCA, Republic Central Africa. Species abbreviations: Colaf, *Coleura afra*; Epogam, *Epomophorus gambianus*; Epospe, *Epomophorus* species; Hypmo, *Hypsignathus monstrosus*; Mto, *Myonycteris torquata*; Rae, *Rousettus aegyptiacus*. Sample abbreviations: s, spleen; l, liver; k, kidney; lu, lung; si, small intestines; fc, feces; u, urine.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HF679391_bPMV_RC09_37l_Mto
HF679396_bPMV_RC10_1893s_Mto
HF679394_bPMV_RC10_1893l_Mto
HF679393_bPMV_RC10_1893k_Mto
HF679395_bPMV_RC10_1893lu_Mto
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0.99
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0.9
0.66
0.72
0.7
0.3

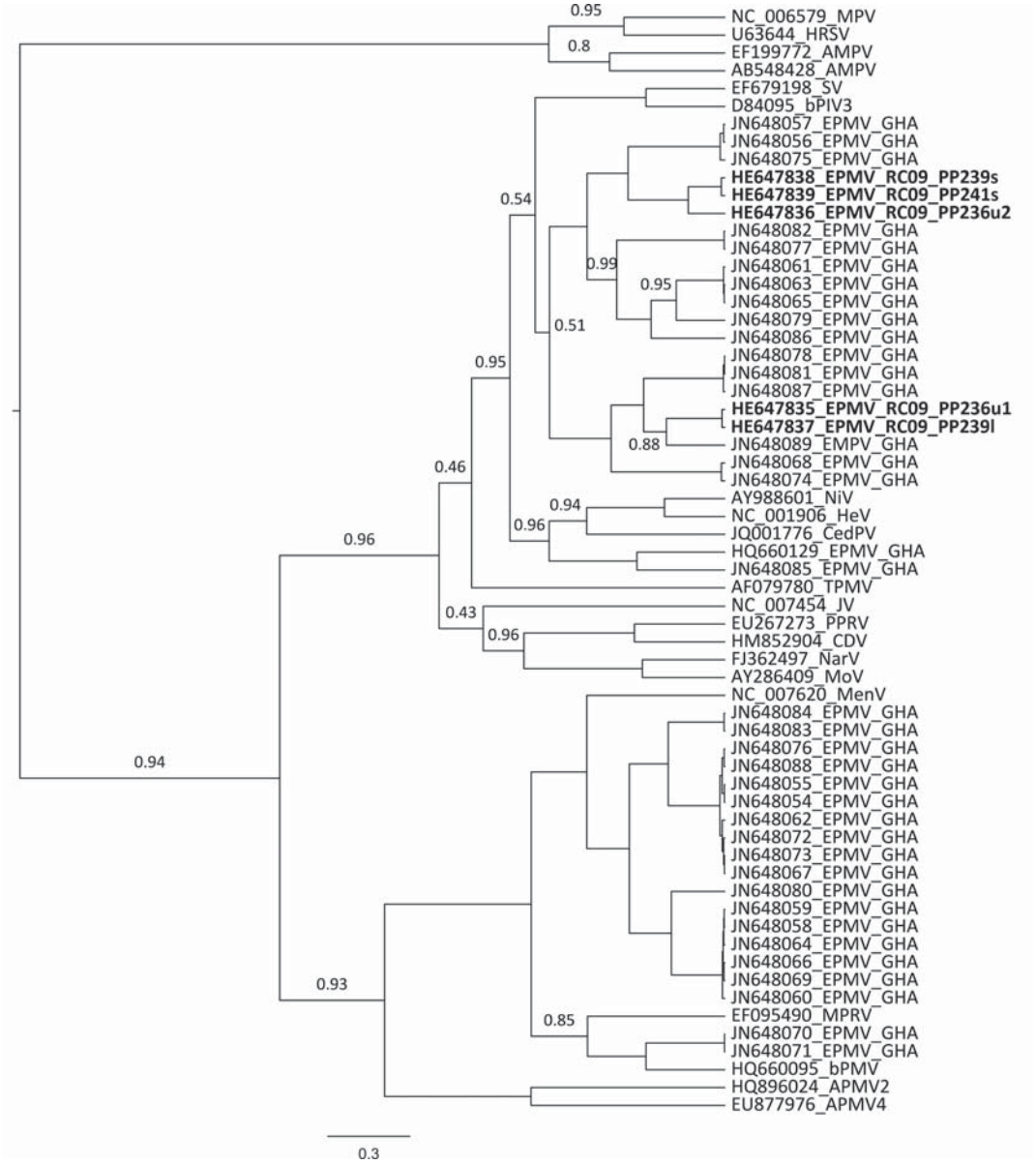

Figure A.5: Phylogenetic tree showing the placement of Eidolon paramyxovirus (EPMV) sequences in the diversity of *Paramyxoviridae*, based on a partial large gene sequence (473 nt) of the *Paramyxovirinae* (PAR) fragment. Trees were computed by using BEAST version 1.7.1 under the assumption of a relaxed, uncorrelated lognormal clock and the Yule process speciation model. Values given are posterior probabilities (pp). For better visibility, only pp values below 1 are indicated. Scale bar indicates nucleotide substitutions per site. Paramyxovirus sequences retrieved in this study are printed in bold and named according to the following pattern: virus_ origin and year_ individual field ID and sample_ species. APMV, *Avian paramyxovirus*; AMPV, *Avian metapneumovirus*; bPIV, *Bovine parainfluenza virus*; CDV, *Canine distemper virus*;CedPV, *Cedar paramyxovirus*; FDLV, *Fer-de-lance virus*; HeV, *Hendra virus*; hPIV, *Human parainfluenza virus*; HRSV, *Human respiratory syncytial virus*; JV, *J-virus*; MenV, *Menangle virus*; MoV, *Mossmann virus*; MPRV, *Mapuera virus*; MPV, *Murine pneumonia virus*; NarV, *Nariva virus*; NiV, *Nipah virus*; PPRV, *Peste-des-petits-ruminants virus*; RPV, *Rinderpest virus*; SV, *Sendai virus*; TioV, *Tioman virus*; TPMV, *Tupaia paramyxovirus*.

A.5 Bat samples tested for antibodies against filoviruses

Table A.2: Bat blood samples tested for antibodies against filoviruses. Samples were tested in an antibody-capture ELISA for binding with cell lysates of Ebola (EV) and Marburg virus (MV) infected cells. PNT=Parc National de Taï, Côte d'Ivoire; PNOK=Parc National d'Odzala-Kokoua (including samples from Brazzaville), Republic of Congo; IFO=Industrie Forestière d'Ouesso, Republic of Congo; GOLA=Gola National Park, Sierra Leone. *fruit- and nectar-feeding bats

Site (year)	**PNT (2006)**			**GOLA (2009)**			**IFO (2010)**			**PNOK (2009)**			**total**		
		reactive			**reactive**			**reactive**			**reactive**			**reactive**	
Genus	**total**	**EV**	**MV**	**total**	**EV**	**MV**	**total**	**EV**	**MV**	**total**	**EV**	**MV**	**total**	**EV**	**MV**
Casinycteris sp.	n.a.			n.a.			1			4			5	0	0
Chaerephon sp.	4			n.a.			n.a.			n.a.			4	0	0
Eidolon sp.*	1						n.a.			33			34	0	0
Epomops sp.*	15	4	4	14	1		12			21		2	62	5	6
Glauconycteris sp.	n.a.			6			n.a.			1			7	0	0
Hipposideros sp.	n.a.			91	4		4			3			98	4	0
Hypsignathus sp.*	11	2	2	23	6	5	n.a.			36	3	4	70	11	11
Kerivoula sp.	n.a.			2			n.a.			n.a.			2	0	0
Megaloglossus sp.*	14		1	13			1			7			35	0	1
Micropteropus sp.*	n.a.			n.a.			n.a.			40	1		40	1	0
Mops sp.	26			4	1		3	1	1	4			37	2	1
Myonycteris sp.*	159	2	6	24	1		52		1	72			307	3	7
Myopterus sp.	n.a.			n.a.			1			n.a.			1	0	0
Myotis sp.	n.a.			5			n.a.			n.a.			5	0	0
Nanonycteris sp.	7			5			n.a.			n.a.			12	0	0
Neoromicia sp.	n.a.			2			n.a.			n.a.			2	0	0
Nycteris sp.	n.a.			8			2			1		1	11	0	1
Otomops sp.	1			n.a.			n.a.			n.a.			1	0	0
Pipistrellus sp.	n.a.			10			1			1			12	0	0
Rhinolophus sp.	1			5			1			n.a.			7	0	0
Rousettus sp.*	n.a.			n.a.			2	2	2	n.a.			2	2	2
Saccolaimus sp.	1			n.a.			n.a.			n.a.			1	0	0
Scotonycteris sp.*	10			8			n.a.			n.a.			18	0	0
Total	**250**	**8**	**13**	**220**	**13**	**5**	**80**	**3**	**4**	**223**	**4**	**7**	**773**	**28**	**29**
(%)		**(3.2)**	**(5.2)**		**(5.9)**	**(2.3)**		**(3.8)**	**(5.0)**		**(1.8)**	**(3.1)**		**(3.6)**	**(3.8)**

A.6 Sequence data of *Taï Forest reovirus*

TFRV, Segment 1

```
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```

TFRV, Segment 2

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5'- attaatgttg  tgagtaccgt  atgtgagtaa  cataatgcca  agaaagaacc  agctaaagtc
    gayaaaagag  acagacaaag  acaaccaaca  gaaaacagaa  cagaccacag  agacagaccc
    aaagaatgtt  ccagggacta  gcaccgccga  tagtggcgcg  agagatgata  aaaatgaaaa
    gaaggtggga  gacgcgagga  acgatgatgc  tgtggcttct  ggtagtagtt  cggataaagc
    gacgagttcc  caaacgggcc  ctgaaaagaa  agatgaagta  cgtgagaaca  gagaaaggaa
    ggtgggtgcg  ctgagagatt  tactgggtaa  ggtatctgaa  gcggttccag  caaaacaagc
    gggcttgcta  agtacgacga  ttccagatga  cgcgttggag  gatatgctca  gagtgttaaa
    agagtttacg  acgttgtcta  aaattgtgcc  atctgcttcg  acttcattag  atcccaatga
    tgtgattcaa  gctgtagttc  ctgaaccttc  tgttggggtt  aagcctgcac  cattaactgt
    tgaggtatta  acatcactac  cacatttcta  caaattgaaa  gtaaaacgag  tagggacatt
    tggtcagatc  atgggtgcat  tctcaggagg  ggattcagct  gataggtttc  cattctcgaa
    ggcaatgaaa  gaggcgttcg  ggtcagggtt  tgattacagc  tacgtggcga  ttgaaactga
    aaactttggc  tttgacttga  agattgatcc  tttaaacctg  catcaagtga  gctgtaaagt
    tgctggaaag  gaggctttgg  gcattggact  gaccgactgt  acaaccgcgc  ccgagatcaa
    gtataatgat  atcaagagtc  cgacagagat  gatgcgtttt  ataatttttc  tgatcgccgg
    tgcggcaaga  actggacgay  ggatatatat  cgccaaattt  gagaacgggg  agaagaagtt
    gttcaatgat  tggttaaaag  taccaggtgt  ggaagccttc  caaaggatag  cgggttggtt
    atgtaacgat  aattacacgg  agtatcctca  tgtacccgaa  gcgatgatcg  agtataaccc
    ctaccgatgg  ttccagcaag  aggtgcgtga  aaatatggtg  cataatggat  tagggaccgt
    tgttgacatg  gcggatggtg  gaccgttagt  ggaggcggat  tttaatcgta  agcgtggtga
    gccggtgatg  gcgaatgttg  attttgtgtg  ctccgaggtt  catgcgaatg  taatggacga
    catgttaaat  gagcgaatga  atgtcgatga  ttacatacac  cttctccaaa  tttgtcagct
    gaatgagggg  gatttacacg  gggacagaaa  gaatatcatt  ctgaaacgat  ataggagaaa
    aacgcttgct  cgagagctcg  aaatgaacat  gctgctgatg  tttccttcgg  atcgcattta
    tcaccaaacc  tacaaacaag  cttttatggt  atcaccgaat  ttccggttgc  acgtgatgtc
    tgtattgatg  gagctgtccc  tttcgaagat  ctctgcattt  aagatgatac  cttcggcaca
    aatgctttcc  gcggtagagt  tacgagagaa  tgtggtgtcg  gcgactgcca  gaggagtacc
    gatcatttta  gcggaatggg  gagcgttgtt  gcacatagtt  acgccaaagg  acatttgcga
    gaggatcgtg  tttgacatca  tggctccatt  tgtaaatgct  tgctcgtggt  atgacaacat
    cggggagcct  agtccttcta  agctgctcta  tgcgttgctt  ggtgcgaaaa  ttgcattata
    tttaaatccc  aacttcttcc  actataacca  acacatttac  gcaagaatga  ttcactcgat
    attttcaact  tttttcgcgc  gagaactatc  aattctactg  ggggacagag  ggtatggagt
    tgacaacttt  ggtcaagtta  agaggacttc  agacgcttat  gagattagca  ggtttgagtc
    gggaattgtc  cattttaccc  tacttcgtcc  atttcctgaa  ccgatgagaa  atgcgagacc
    aggtcggtgg  cgcgtgatgg  ttaacattca  aaattggttg  ttagataacc  ggaccgctta
    tcttcatgag  gttggagggt  gtcgatacca  gccagatccg  agacggattc  gtgtaccggg
    gcaaatattt  gtaccacata  ggacgatggg  tgctggagac  ttgccaatat  atggggcctt
    acttggagcg  atcgatctcg  tcgaggagta  taggaatgta  actcgccctg  ctgaaaggta
    ctatcagctg  ccatgggaga  atattgcgat  tgacattctg  atgcatatca  ttactcctta
    tctggtgtct  ttctctgaat  ggtttcactg  ggtgtactgt  ccaatatacc  ttcaagtggc
    ccttcatcct  atgatttggt  ggaatcatga  gaatgatcca  gatgcatggc  gccgatttca
    cgcgttgcga  gagaatccgg  acgaggttat  tagacacaat  ctgagaggtg  tacctttctg
```

```
        10          20          30          40          50          60
         |           |           |           |           |           |
tcatcctgtt  cctgttttgt  ttagtgagaa  agagagcatg  cgtccatttt  cacttcatga
aggattggat  attttgggaa  gagtttatgc  gccgggtagt  acgtgcaagg  gagggaggta
tgataacgat  gctgtaattc  aaattagtga  ctatcacatg  atgtggcaat  caaatgacag
cgagacgatg  gtgagacagt  taacggtggc  aaggtatgga  gggagggaac  tgatggaggg
tcttaagcta  ataggagatc  tgtctttagg  gagactgctt  gataacatgg  ctgttcacga
gtacatccgt  aatgcgttta  tggagtcaag  gcttccgagt  cggacgtact  atgagatttt
agacttatac  gggattaaga  ttgacgatag  agcattggcc  caacttcctg  gaggcttttt
agaagacaaa  ttaatgggag  atttccgttt  gtactttccc  gagactggac  gttatgatcc
aaatggtcga  tttatcccag  atggtcgttt  ggtagttgac  cccgtgagac  gctccgtacg
tgagaatgtt  cggatcctaa  tgaacgccgt  tttcaatgag  gattatggaa  tgattaagtt
caggacggga  atgaccctgt  ctctacagcc  tcagaatgat  ctccttttcc  tatcaacaca
tccacttttc  tccttttacg  ccagaatgac  gggggtagta  agcttcgtac  gtcaagccga
cactgacagg  acggtcatat  cccatgaaat  gattgtagtg  tggacagatc  atagaaatag
aagatgtaat  gaattatttt  ctgtaaaaac  gatatatgat  ctgatggagg  cgctcaggct
gagggttgga  ggcaccgttg  atggggttac  ttttgtggtc  agcgacttac  gaaggatgag
accagagtca  tatcagttta  tacttgacgc  cgtcaatcaa  catctggctg  tagtgtacct
tccaaaagta  agagggatta  tacatcacca  catcactcaa  attactgaga  caacacttat
ttttaaacag  cacgagttga  atatgtttga  acatatgctt  ggaatggctc  cgacggcgtt
agttcagatg  aatgtggtgg  agtcgattcg  tgcaagaggt  ggagttatca  ctagtaatat
tcagaagcct  ttgctccctg  tagatccggt  aactgatcgg  tgggtttttt  gtactgacgt
taatacggtt  gaccgcaggg  ccctcggacg  accggtccgt  attcgagagt  tttttccaga
gattgcacat  cgacatggag  ggagaccttg  gattaggctg  gagaatgatg  aagttgtgcg
tggatatcct  ctgcacccat  acggattgcg  atcaatcata  tt -3'
```

TFRV, Segment 3

```
              10          20          30          40          50          60
               |           |           |           |           |           |
5'- ccacaaaatg  tttcatttcc  agaacagacc  tacagaacag  cttagaacca  gaatcagcgc
    cagaaattat  aaagcagatg  cgtattcgcc  ccataattct  gccaactgtt  aattggaatg
    gacggttaat  cacgtctgga  actgtaggag  ttagttttga  tcatccggtc  tactcgcgaa
    tcattccatc  tctcgtcgca  actgaggtct  atactcacac  cctctccgag  ctactgaagc
    tagagaagaa  tgttgatgaa  gatgttttaa  ggagggttca  gagggcggcg  tgcacagagg
    tggcctatca  gtatattgcg  agaattagtt  cattataccg  cgtgattctt  gaaaactatc
    ctactgattt  cgtttgggca  caggctggcg  agttagtata  cgatgaagtt  tttttgttac
    cggctctgta  tgagattgct  agggatcgag  ccgccaatgt  cttgatgtat  gagggttatc
    atgggtgtat  tggaggtcga  gttgccattg  acggggatcg  gaatgaggga  atgcgacggc
    tttttgtaac  atggttgcat  ggaatgggtc  agatacaagc  tcctgaatat  gaaagtgtta
    tggatcctga  atataaggtt  gttttggagc  gagactataa  ggtgtttttt  cttggggact
    ctttaccgag  agatggtgat  ctaggatggt  tacatgggaa  gatgccctac  aggacggtat
    ggatgcccaa  cgcaagttac  tcgcgaatgg  tgcgtagaat  gttacttagc  aggatgccga
    gtggagctca  tggtgtactt  aacattccaa  ttccccacta  tttcgagttt  gattgtgctg
    cagatttaga  gcttagatgc  tggcatgggg  tccgtgcgac  atggttaaaa  ctatttgatg
    caaacgttaa  cggggaggtt  gatgattgtc  gattgaatga  agctttaatg  gtgaattgtc
    ctcgtgattt  atcatctgct  gtccgagagt  caatgttttc  gtgttcttta  gctagagggg
    tgcctcctca  cgttgtgtct  acattgaaga  gcgttaaaat  gatagctgga  gcgcagcatt
    atcatgttgt  acaaggggac  ggagattggt  ggcgtcggga  agacattttt  cctcaaggac
    ggtacagcat  tagatgggga  gagattatcg  cgattcgtgg  aatagatgag  gggattccgg
    atagatttgc  gagttggata  gaggtgatga  atccgattta  ttataccgac  attggattga
    aggctaacca  gctaatgata  actgtcccta  ctgatcccga  gccagggtgg  attgaagtcc
    atgagaatga  cttgaagttt  aaggtgtatc  aattggacag  agtgaactat  tccgttgatc
    cagtgagttc  ttgcgcgaca  atcttctttt  ctcaagttca  atttgattta  ccgtttccgc
    caccctacga  gttggatcaa  cagagagatg  cagtggtaaa  tcgtgagctg  tataatgcga
    tgactcttgg  tagtaaccgt  caaattggcg  agtttcacgg  aaagattgct  tcgaccacta
    agaagttaag  ggatctgtat  acattcgaga  aggacagaac  ggttccggtt  aataccgcat
    taaacagaaa  ggatgcaaaa  gagaatccgc  ttgaatatct  ctgtgttgaa  gcgtggattg
    aggctctgcg  tactgtaagt  gaccaaatgg  attatgatcc  gagatgggca  cacgatgatc
    aggaacaaga  tgatcctaga  tatcgattgg  tggatgttat  gattcgtgtc  tcaaagcact
    tgtggtctaa  accttacaga  ctgtcaatga  tcgcaaacgc  aaccttatta  attcatgcga
    ttgctcgaca  cttgttaagt  tttaacttag  ctcactatga  tggtttgaga  ttagaggatt
    taaccattgc  gtggtatggt  gagggtgcgg  aggagatgac  tcgattaatg  aaggagtgtg
    gttttaaaag  atacattccg  atacctaata  gtatgggggg  agctgatcat  atggagcagc
    atgttttgcg  gggagtcgtc  gtg -3'
```

TFRV, Segment 9

```
              10          20          30          40          50          60
               |           |           |           |           |           |
5'- tgccccctct  ctaaatcctg  caccttcgat  gttcaccaga  aacaactcac  caggccaacc
    ccgtcaacaa  cagcctcgta  taagtttttc  ctgactaaca  atcaaactct  ccatcaaccc
    aattagttct  ctccatgcca  aatcaaccgc  ggcctgcctc  ttatctgaca  ttactgtacc
    ggtatacaaa  ggacctgccc  taattctcgc  ctcccacctc  tcatcattcg  tatttcgttc
    cttctgttcc  acgatcacct  ctgggacagg  taggccatga  gcatcacaaa  gctctcttcc
    tagttcttca  gcctgaccgg  caccaataac  tctcttctta  ccttccgcct  gtgacatcat
    tgcaaacact  gctgcatcat  acgacttccc  aagcaatggg  tgtgctctag  ttatgaaatt
    gaatccgcgg  ataaatttcg  caactgtctc  attatcgtcc  tgcacccaat  ccaactcttt
    agccgtccca  tatactaaca  aataccttac  cgtcagcttt  ctctcataag  gaatataata
    catggggccc  ttcgccgtca  atccatcata  tgttcgttta  caatccattc  tcacaattaa
    aggaccagtc  acgttatcct  tgatccattg  ccatgcttct  gacgcaggaa  tctccctttt
    caaagcagtg  tgtataaatt  tcgccggctc  atgcatgtac  aacacaattt  tatccttatc
    tccacgcata  ttataagcct  cctggaacgc  atcgaatcgc  acctctgtct  ttgctctcat
    ccatgtaggt  gtagaatcag  ttctagctaa  tgctccaacg  tttgtcccaa  taaaagcata
    gttcccaaca  tcaccaaata  ccaactgagc  atgagacaca  gtgacatcgc  aataatctcc
    aagccaaagc  agcttacaat  catcaccaaa  atggacttta  atagatcgca  taacaatcag
    atcacgccgt  gtcgggtcat  agcccctctg  ggaaagtgat  acagggtagt  tcccaaagtc
    tttaatgtat  aggtcggttc  gtgtgttgat  tgcctgtaaa  tatccgaaca  tagtgtatgt
    ggaggtcgct  agatggtca   -3'
```

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Selbständigkeitserklärung

Ich erkläre an Eides Statt, dass die vorliegende Dissertation in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind. Veröffentlichungen von Teilen der vorliegenden Dissertation sind bei den Referenzen angeführt. Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Berlin, den 07.03.2013

Sabrina Weiß