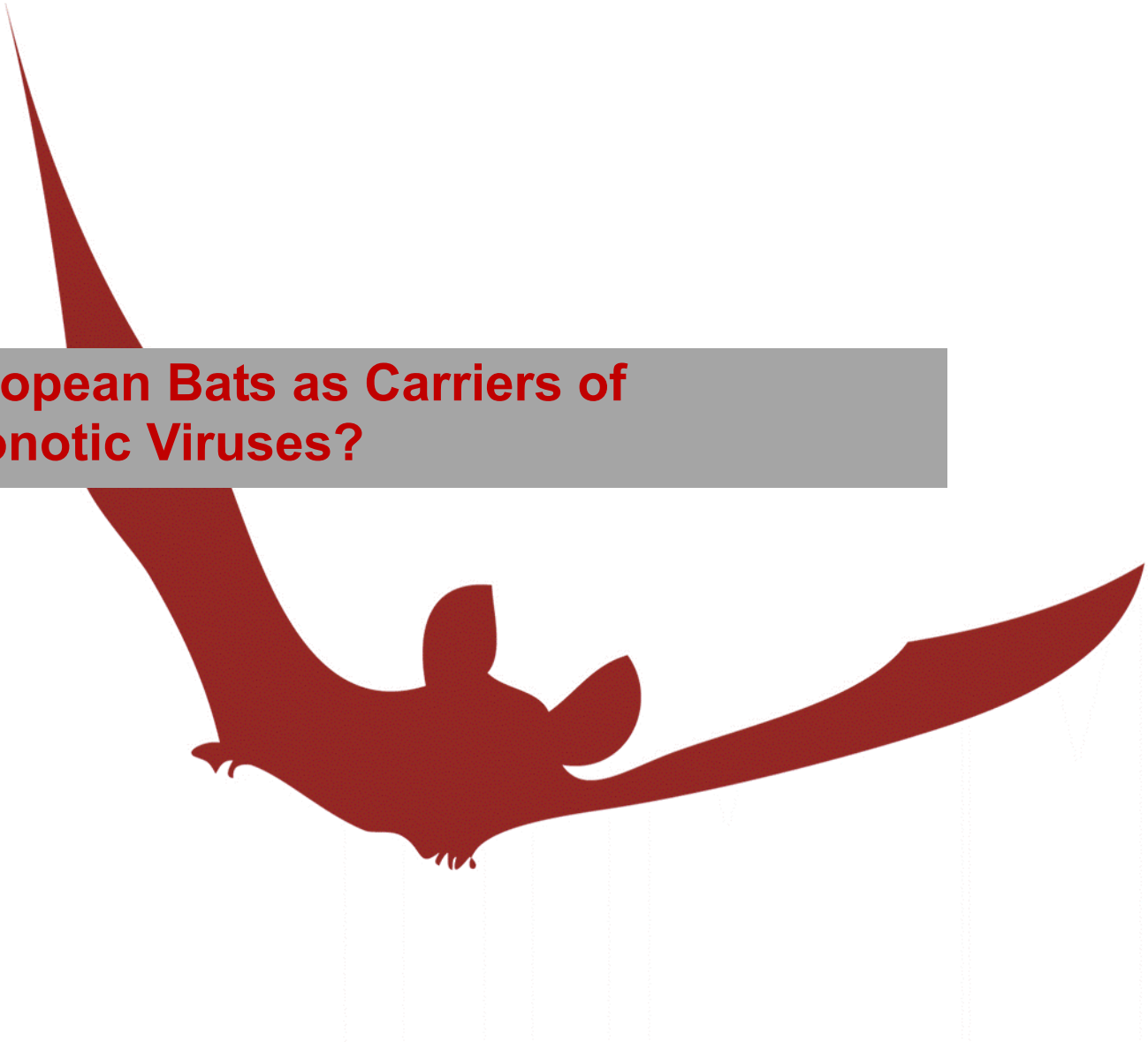
European Bats as Carriers of Zoonotic Viruses?



Claudia Kohl | Prüm

“There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened.

Douglas Adams “The Restaurant at the End of the Universe”

European Bats as Carriers of Zoonotic Viruses?

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DECLARATION

Gemäß § 7 Abs. 4 der Promotionsordnung vom 04.09.2007 des Fachbereichs Biologie, Chemie, Pharmazie der Freien Universität Berlin versichere ich hiermit, dass ich die vorliegende Dissertationsschrift mit dem Titel „*European Bats as Carriers of Zoonotic Viruses*?“ selbstständig und ohne unerlaubte Hilfe angefertigt habe.

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ACKNOWLEDGEMENT

TABLE OF CONTENT

ACKNOWLEDGEMENT ..... **I**

TABLE OF CONTENT ..... **III**

1 INTRODUCTION **1**

1.1 BATS 1
1.1.1 Unique Features of Bats *1*
1.1.2 European Bats *4*
1.1.3 Evolution and Diversity of Bats *4*
1.2 BATS AS VIRUS RESERVOIR HOSTS 8
1.2.1 Zoonoses and Disease Emergence *8*
1.2.2 Key Examples: Bats Worldwide as Potential Reservoir Hosts *9*
1.2.3 Viruses in European Bats *14*
1.3 VIRUS DETECTION AND CHARACTERIZATION METHODS 19
1.3.1 Virus Detection *19*
1.3.2 Next Generation Sequencing *21*
1.3.3 Sequence Analysis in silico *24*

2 STUDY DESIGN **28**

2.1 EXAMINATION OF EUROPEAN BATS FOR THE PRESENCE OF SPECIFIC PATHOGENS 28
2.1.1 Description of the Comprehensive Study *28*
2.1.2 Virological Examination at the RKI *29*
2.1.3 Aims of this Thesis within the Comprehensive Study *31*

3 SUMMARY OF RESULTS **33**

3.1 PUBLISHED RESULTS OF THIS THESIS 33
3.2 PARTICULAR CONTRIBUTION TO THE MANUSCRIPTS 34
3.3 ABSTRACT MANUSCRIPT I: 35
3.4 ABSTRACT MANUSCRIPT II: 35
3.5 ABSTRACT MANUSCRIPT III: 36
3.6 ABSTRACT MANUSCRIPT IV: 37
3.7 ABSTRACT MANUSCRIPT V: 38

4 DISCUSSION **39**

4.1 VIRUS SCREENING AT THE RKI 39
4.1.1 PCR Screening for Zoonotic Viruses Strongly Associated with Bats *40*
4.1.2 PCR Screening for Other Highly Pathogenic Viruses and Relatives *44*
4.1.3 Conclusive Statement to PCR Screening *50*
4.1.4 Virus Isolation *52*
4.1.5 Conclusive Statement to Virus Isolation *55*
4.2 VIRAL METAGENOMICS 55
4.2.1 Development of the TUViD-VM Protocol *56*
4.2.2 Preliminary Results: Bat Viral Metagenomics *58*
4.3 RELEVANCE AND RISK ASSESSMENT FOR EUROPEAN BATS 59
4.3.1 Principle of Cause-and-effect: The Henle–Koch Postulates *59*
4.3.2 Bats as Reservoir Hosts *60*
4.3.3 Zoonotic Bat Viruses in Europe? *63*

5 CONCLUSIONS AND FUTURE PROSPECTS **69**

5.1 CONCLUSIONS 69
5.2 FUTURE PROSPECTS 70

6 ABSTRACT **71**

7 ZUSAMMENFASSUNG **72**

8 TABLES AND FIGURES 73

8.1 TABLES 73
8.2 FIGURES 73

9 LITERATURE 74

10 LIST OF PUBLICATIONS 85

11 CONTRIBUTIONS TO CONFERENCES 86

12 CURRICULUM VITAE 87

13 APPENDIX 88

13.1 TABLE A1: CHARACTERISTICS OF EUROPEAN BAT SPECIES 88
13.2 TABLE A2: VIRUSES FOUND IN EUROPEAN BATS 93
13.3 MANUSCRIPTS 96
13.3.1 Manuscript 1 96
13.3.2 Manuscript 2 102
13.3.3 Manuscript 3 110
13.3.4 Manuscript 4 118
13.3.5 Manuscript 5 130
13.3.6 Manuscript 5 Supplement 144

1 INTRODUCTION

1.1 BATS

Bats are the second largest order of mammals after rodents and are composing about 20 percent of all extant mammals in the world. They can be found throughout the world except for the polar regions, inhabiting not only rural, but also urban environments. They play a major role in insect control and plant pollination (Neuweiler, 2000).

Bats are homeothermic, furred, have external auricles, give viviparous birth and lactate their pups (Dietz et al., 2007). Combined with their typical teeth formation, they are evidently belonging to the class of mammals. However, bats have developed evolutionary adaptations, unique for bats and very untypical of the remaining species of the taxonomical class.

1.1.1 Unique Features of Bats

1.1.1.1 Ability to Fly

While other mammals (gliders and flying squirrels) developed membranes between their arms and legs, allowing them to glide between trees, bats are the only mammals capable of active wing beat and flight (Neuweiler, 2000). Their elongated fingers serve as a wing scaffold, and the flight membrane (*patagium*) forms the wing shape around them, enveloping body, extremities and tail. All mammals showing this phenotype (i.e. all bats) are consolidated to the order *Chiroptera* ('hand-winged') and are divided into the suborders *Micro-* and *Megachiroptera*. The largest wing-span registered worldwide (1.7 m and body weight of up to 1.5 kg) is reached by the *Megachiropteran* bats of the genus *Pteropus*. In contrast, the smallest bat *Craseonycteris thonglongyai* merely reaches a weight of 3 g and a wing-span of 13 cm (Dietz et al., 2007).

1.1.1.2 Hanging

All bats have in common that they rest while hanging upside (head) down. This might be an adaptation as a consequence of bone-weight reduction necessary for sufficient flight quality. Compared to birds, mammals have rather compact bones: bats' leg bones and muscles have adapted to a more bird-like lime-bone structure, probably resulting in reduced stability (Swartz et al., 1992). Comparing the physiology of bats and birds, one needs to take into consideration that they have evolved independently. They are showing a convergent evolution regarding the ability to fly, but the mechanisms allowing them to fly are very unlike. Their hanging behavior is also energy efficient as sinews in their toes can get locked, allowing for fatigue-proof hanging during rest and hibernation (Neuweiler, 2000).

1.1.1.3 Longevity

The longevity of animals – although not universally accepted (Austad, 2010) – is correlated with their body size and metabolism rates (Lindstedt and Calder, 1981). In contrast, some bats have an exceptionally long life span when compared to other mammals of the same size (*Myotis lucifugus*, 6-10 cm body size, 34 years in the wild). One explanation of an exceptionally long life span could be their settling in an ecological niche, which prevents them best from possible predators (Dietz et al., 2007). Due to their ability to fly and their nocturnal way of life, only few predators are specialized on bats.

1.1.1.4 Mating and Birth

Most bat species give birth once a year to one individual. There is an exceptional mechanism how bats can influence the time point of fertilization and birth: For instance, European bats mate during summer or the beginning of autumn. The semen, which has great viability, is then 'stored' during the winter months in the uterus of the female until ovulation in spring, after hibernation. Moreover, the duration of gestation is flexible and dependent on food availability, between 40 and 70 days for European bats (Dietz et al., 2007; Neuweiler, 2000).

1.1.1.5 Torpor and Hibernation

Torpor and hibernation has developed in bats convergent to other hibernating mammals. Torpor describes the controlled reduction of the body temperature to a distinct level in between ambient temperatures and the usual 37°C body temperature (Audet and Fenton, 1988) to save energy in months of limited foraging by a reduced metabolic rate. Since torpor is an active mechanism, the body temperature can in turn be elevated quickly in case of disturbances, but on the expense of the energy stored in the bats' brown adipose tissues. In the summer months, torpor is always a cost–benefit calculation for the bat: Not only is the metabolism depressed, but also the reaction rate of the central nervous system is reduced, which results in reduced safety of their roost whereas maintaining lower food requirements. In addition, pregnant bats need to balance the faster development of the embryo at higher temperatures against energy saving (Willis et al., 2006). In the winter months, the latter has certainly the highest priority which leads to hibernation, in which bats lose up to 40 percent of their initial weight, being a risk in the case of cold and delayed springs (Dietz et al., 2007).

1.1.1.6 Echolocation

Probably the most famous attribute of bats is the ability of echolocation. Nearly all members belonging to the suborder *Microchiroptera* use echolocation, whereas most members belonging to the suborder *Megachiroptera* navigate with specialized nocturnal vision (Dietz et al., 2007; Norberg and Rayner, 1987). Interestingly, phylogenetic sequence analysis showed that the

prestin gene, responsible for high frequency sensitivity and echolocation in the mammalian auditory system, has evolved convergent between both unrelated lineages of bats and dolphins (Liu et al., 2010).

1.1.1.7 Immune System

Bats are suspected to be reservoir hosts of numerous viruses, however, studies comprising bat immunity are rare. Bat infection studies have resulted consistently in the lack of clinical signs in bats, although viruses have replicated efficiently in the bats' organs. Recently, several hypotheses of special immunological features in bats were published (Baker et al., 2013b; Leroy et al., 2005; Middleton et al., 2007; Pourrut et al., 2009; Swanepoel et al., 1996; Towner et al., 2009) and consecutive studies have focused on the innate and adaptive immune response of bats.

A possibility of why bats remained apparently asymptomatic to many virus infections might originate in their innate immune response. The innate immune system comprises mechanical barriers, immune cells (lymphocytes, macrophages and granulocytes), the complement system, interleukins and pattern recognition receptors (PRRs) (Kawai and Akira, 2006). Even though limited bat-specific methods are available to study their cell types, studies identified a variety of immune cells (i.e. macrophages, dendritic cells) very similar to those of other mammals based on their morphological structures (scanning electron-microscopy) and biochemical functions (cellular adherence) (Baker et al., 2013b; Sarkar and Chakravarty, 1991; Turmelle et al., 2010).

PRRs (i.e. toll-like receptors) are the frontline defence against infections. Toll-like receptors have been described as highly conserved in bats, in particular compared to mice and other mammals (Cowled et al., 2011).

Interferon (IFN) I (α and β) and III (λ) are directly involved in the defence of viral infections (Kawai and Akira, 2006). Interferon I described in various bats is encoded in only seven IFNα genes, some of which are only pseudo genes (Baker et al., 2013b). In contrast, humans and mice have 13 or 14 IFNα genes.

The complement cascade of bats showed differences between the single species: while the complement efficiency of tropic fruit bats decreased at temperatures below 37°C, the complement activation in the tested microbats was overall more efficient and insensitive to changes in temperature. Microbats hibernate, and though it might be a biologically necessary adaption of their complement system, affecting the overall function of the complement cascade (Allen et al., 2009).

The adaptive immunity comprises B- and T-cells, immunoglobulins and cytokines. It has been demonstrated that bats transcribe immunoglobulin (IgM, IgE, IgA and multiple IgG) classes,

homologous to human Igs (Butler et al., 2011). The cell-mediated adaptive immune response is controlled by cytotoxic T-helper cells, but is not yet fully characterized in bats. Still, studies describe a delayed T-cell response in bats. MHC I and II and cytokines (interleukin and tumor necrosis factors) have partly been characterized (Baker et al., 2013b). The comparative analysis of two bat genomes (*Myotis davidii* and *Pteropus alecto*) revealed a high concentration of positively selected genes responsible for DNA repair and the immunological pathways that might have developed during the evolvement of their flight ability (Zhang et al., 2013). In the latter study, the authors suspected that the ability to fly and the functions of major immune responses and aging in bats evolved independent of each other.

1.1.2 European Bats

The weight-span of European bats comprises 5 g in *Pipistrellus pipistrellus* and 50 g in *Nyctalus lasiopterus*, the largest European bat. European bats prey on insects, but *Nyctalus lasiopterus* sometimes have been reported to prey on smaller birds as well. The European species that inhabit colder regions use torpor and hibernation; many migrate over vast distances while others are rather territorial (Appendix Table A1: Characteristics of European Bat Species). All bats in Europe are utilizing some kind of echolocation to navigate, albeit the Egyptian rousettes (*Rousettus aegyptiacus,* prevalent in Cyprus, Tenerife and Southern Turkey) use a more primitive click-sound echolocation (Dietz et al., 2007).

1.1.3 Evolution and Diversity of Bats

The oldest known bat fossil dates to 52.5[1] Ma (Wyoming, USA and Dessel, Germany) (Jepsen, 1966). The *Paleochiropteryx* (preserved in stone) showed two interesting attributes: Butterfly scales were found in its intestinal tract, leading to the assumption of ancestral insectivorous feeding behavior. In addition, the ancestral *Paleochiropteryx* exhibited an additional claw on its index finger; a physiological attribute that can be seen nowadays in *Megachiroptera* only.

Today, about five events recorded in marine fossils are known that caused mass extinction during the history of earth geological periods: late Ordovician (485 Ma), late Devonian (420 Ma), late Permian (250 Ma), late Triassic (200 Ma) and end Cetaceous (66 Ma) (Raup and Sepkoski, 1982). The latter is known as the Cretaceous-Paleocene (K-Pg) extinction event which is responsible for the disappearance of the non-avian dinosaurs and approx. 70 percent of all species in the world, a fact that is proved by fossil records (Molina et al., 2004) (Figure 1). The

[1] Ma = Megaanum - Unit of time equal to one million years.

effect of the incident is assumed to have long been overestimated regarding the subsequent development of biodiversity. Nowadays, studies are showing molecular-based evidence that the K-Pg extinction event has not been the fuse of species diversification on the family level. This had apparently already happened more than 30 Ma before the K-Pg extinction event. But the species diversification of animals that were not obliterated by the K-Pg extinction event progressed more rapidly. Animals that survived the K-Pg extinction event on a population level share the ability to take shelter in soils, underground, in rock piles or tree holes to survive the heat blast and subsequent nuclear winter caused by the K-Pg extinction event (Wang et al., 2011). In the aftermath, new niches had become available due to extinction of biological richness. The survivors filled the gaps rapidly during the early Eocene, resulting in a short period of divergence and adaptive radiation to their novel niches (Simmons, 2005a).

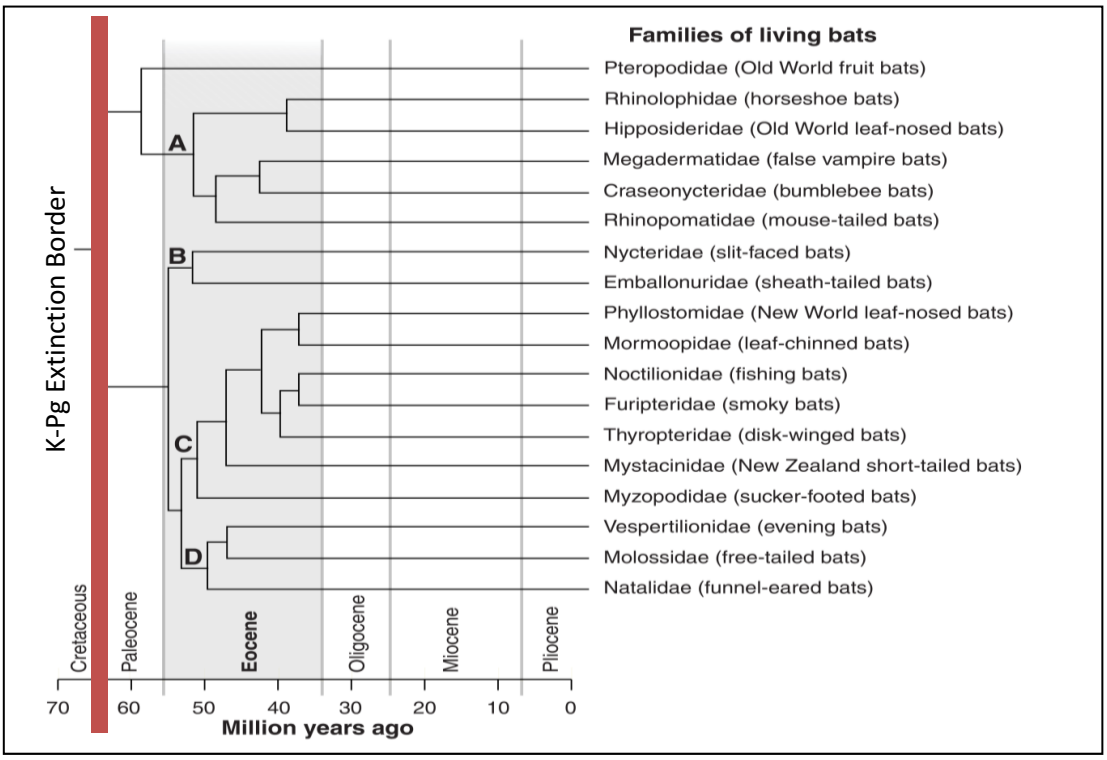

Figure 1 Temporal pattern of bat diversification [adapted from (Teeling et al., 2005)]. A molecular tree was generated, reconstructing the time of bat radiation with the help of fossil calibration points. Extant bats shared a common ancestor at or just after the Cretaceous-Tertiary boundary (K-Pg; indicated in red).

Alexander von Humboldt discovered the latitudinal gradient in species diversity as early as 1799 (Humboldt, 1826): The richness of species is subject to a global diversity gradient, abating from the species-rich tropics toward the higher latitudes (Figure 2, Figure 3) (Buckley et al., 2010). Bats influence this gradient significantly by increasing species richness (Figure 2), resulting in a four times steeper worldwide diversity gradient when bat species are included (Buckley et al., 2010). The authors assumed a link between temperature and energetic costs for bats,

resulting in a distribution predominantly in the tropics. By specialization and changes in metabolism, thermoregulation was assumed to be obsolete and no longer suitable for temperate regions. However, occasional niche shifts resulted in thermo regulative adaptations, allowing bat species to radiate beyond the tropics towards temperate climates (i.e. Europe), resulting in the diversity gradient we see today.

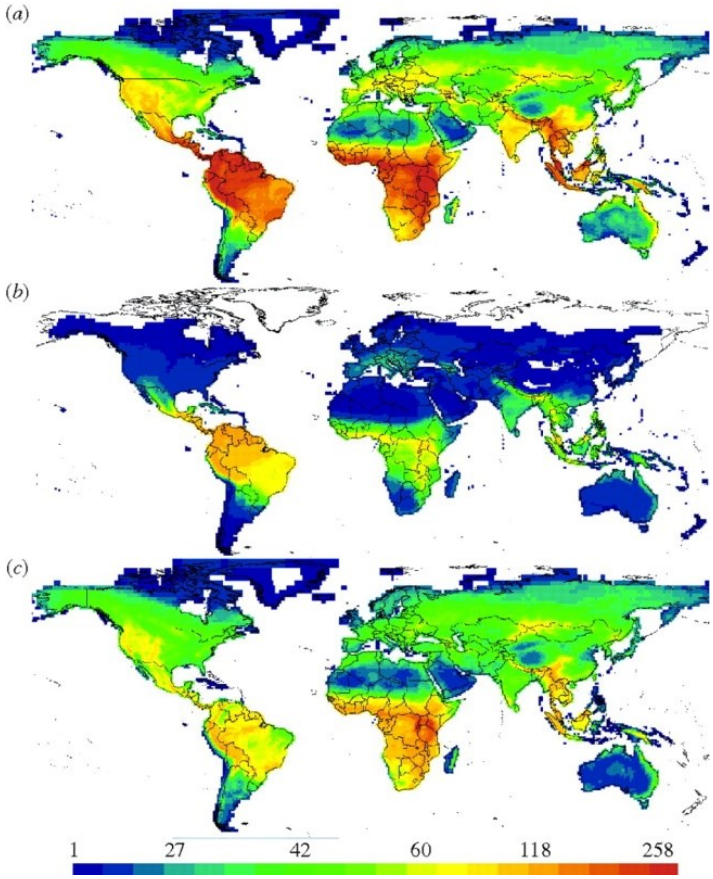

Figure 2 [adapted from (Buckley et al., 2010)]. Species richness map for (a) all the species, (b) bats only and (c) all species other than bats, showing how bats influence the overall latitudinal diversity gradient. Data are depicted as 20 quantiles based on the data for all mammals, with warm colours indicating greater richness.

Like no other mammals, bats found ecological niches in every environment of the planet, except for the oceans and polar regions, and are highly abundant: e.g. Israeli desert-dwelling bats, red flying foxes adapted to the Australian outback, tropical bats, bats living in temperate climate, bats roosting in caves, trees, buildings, roofs and even living in carnivorous plants (Grafe et al., 2011; Nowak, 1994). Bats' feeding behavior is likewise diversified: Insectivorous, frugivorous, nectarivorous, hemovorous, omnivorous, carnivorous and herbivorous species and moreover either an obligate or facultative diet. Their social behavior ranges from a solitary existence to vast, highly sociable colonies.

For a long time the evolution of bats has been discussed controversially. On the one hand, scientists did not believe that the evolution of the nocturnal eyesight and distinct brain areas of

the *Megachiroptera* was convergent to the considerably similar vision of nocturnal monkeys (lemurs); on the other hand, scientists assumed the origins of *Megachiroptera* to lie within the *Microchiroptera* and ancient tree shrews. Eventually, molecular-genetic approaches shed light on the molecular evolution of bats that led to their unique diversity (Jones et al., 2005; Simmons, 2005b; Teeling et al., 2005). Similarly, the evolutionary relationships within the distinct bat families were often subject of discussion until molecular-genetic analysis revealed these relationships. Until then it has been widely assumed that the more visual navigating megachiropteran bats were phylogenetically distinct from echolocating microchiropteran species. Genetic data, however, revealed the split of the *Chiroptera* into two subclades: the first subclade *Pteropodiformes* comprising the optically navigating megabats (*Pteropodidae*) and, surprisingly, the echolocating horseshoe and horseshoe-like microbats (Figure 1 A). The second subclade *Vespertilioniformes* comprised the majority of echolocating bats (Figure 1 B,C,D) (Teeling et al., 2005). This provides two differing evolutionary scenarios: Either echolocation evolved convergent in both subclades *Pteropodiformes* and *Vespertilioniformes* or the whole family *Chiroptera* formerly displayed echolocation, but only the *Pteropodidae* lost their ability to echolocate during specialization on fruits, while insectivorous bats brought their echolocation to perfection.

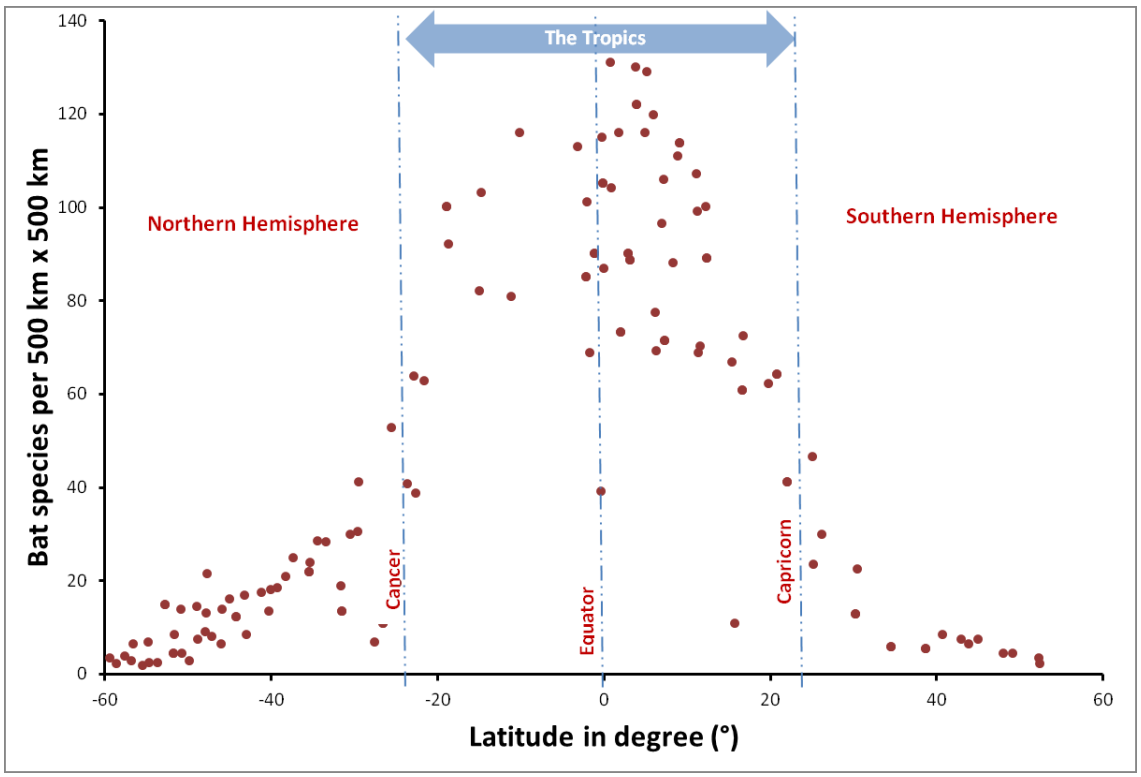

Figure 3 Number of bat species per 500 km x 500 km, correlated to the latitude. Adapted from (Dietz et al., 2007; Willig and Selcer, 1989).

1.2 BATS AS VIRUS RESERVOIR HOSTS

1.2.1 Zoonoses and Disease Emergence

1.2.1.1 Disease Emergence

Fifteen millions of approximately sixty million human deaths worldwide per year (25 percent) are presumably induced by infectious diseases (Morens and Fauci, 2013; Morens et al., 2004) caused by microorganisms (bacteria, viruses, parasites, fungi, prions). We can distinguish three kinds of infectious diseases dependent on the range and period of emergence (Fauci and Morens, 2012): *Endemic infectious diseases*, prevalent during a certain period of time and resulting in predictable levels of diseased individuals (e.g. respiratory infections); *reemerging infectious diseases*, historically known to cause diseases in humans but assumed to be under control before they reemerged (i.e. MRSA); and *emerging infectious diseases (EIDs)*, recognized as pathogenic for the first time (i.e. *Severe Acute Respiratory Syndrome* Virus, SARS-CoV) (Fauci and Morens, 2012).

Infectious diseases have a distinct role in the panel of human diseases. On the one hand, disease outbreaks are unpredictable in making a vast global impact and pathogens are adaptable to their hosts and can be highly transmissible and lethal. On the other hand, infectious diseases bear the possibility of straightforward prevention measures, containment and possible eradication (e.g. through vaccination or hygiene). Outbreaks of infectious diseases are assumed to reflect our relationship with the environment, our interaction with animals, our behavior and our social interactions (Fauci and Morens, 2012). Emerging infectious diseases are nevertheless not suddenly fulminating out of nowhere, but have to come from some source, often animals.

1.2.1.2 Zoonoses

Venkatesan et al. described zoonoses as human diseases caused by animal pathogens or animal diseases transmissible to humans (Venkatesan et al., 2010). They can be transmitted between humans and animals either directly or indirectly: Direct transmission involves contact between the infectious animal and a susceptible human host through e.g. animal bites, handling of animals or handling of clinical specimens. Indirect transmission encompasses vector-borne transmission, for instance by hematophagous arthropods (mosquitoes, ticks, sand flies and midges).

A prerequisite of infecting a human being (or another animal species) is the ability of the pathogen to overcome the species barrier. The species barrier itself comprehends all factors that make the differences between the species on physiological and molecular levels (Pepin et al., 2010). Selection pressure accelerates the evolutionary adaptations to overcome these barriers.

For instance, such pressure can result from anthropogenic influences on the environment: Deforestation of the rainforest changes and destroys the habitats of residential animal species. As a consequence, the resident animals have to relocate more closely to the anthroposphere or to habitats overlapping with those of other animal species. This increases the likelihood of animal–human or animal–animal contacts and thereby raises the chance of inter-species transmissions. Apart from this, many further drivers of disease emergence exist; all of them have in common that they originate from human overpopulation, increased human mobility and the depletion of natural resources.

1.2.2 Key Examples: Bats Worldwide as Potential Reservoir Hosts

Various publications reviewed bats worldwide as carriers and potential reservoir hosts of human-pathogenic and zoonotic viruses (Calisher et al., 2006; Kuzmin et al., 2011; Smith and Wang, 2013; Wang et al., 2011; Wong et al., 2007). In this chapter only the key examples with particular importance are described.

1.2.2.1 Coronaviruses

Coronaviruses (CoV) are divided into four distinct groups alpha-, beta, gamma- and deltacoronaviruses. SARS-CoV and MERS-CoV which are subject to the next paragraphs are belonging to the group betacoronavirus.

SARS Corona Virus

From November 2002 until July 2003 the world was confronted with the first pandemic of the new millennium, caused by a novel coronavirus inducing the *Severe Acute Respiratory Syndrome* in humans (SARS-CoV) (Drosten et al., 2003; Fouchier et al., 2003; Kuiken et al., 2003). The pandemic spread from its origin, a wet-market in the Guangdong province in China, through 33 countries on five continents and resulted in more than 8,000 infected humans of whom more than 700 eventually died (Peiris et al., 2004; Wang et al., 2006).

The genome of the novel SARS-CoV was sequenced and the phylogenetic analysis revealed no close relatedness to any coronavirus known so far (Marra et al., 2003; Rota et al., 2003). The search for the animal reservoir began, identifying masked palm civets as a possible source. However, experimental infections of masked palm civets resulted in the development of clinical signs of infection (fever, lethargy and loss of aggressiveness), which is thought to be an exclusion criteria for a reservoir host (Hudson et al., 2002; Wu et al., 2005). However, civets most likely played a key-role in the initial transmission and spill-over.

Shortly after, horseshoe bats (*Rhinolophus spp*.) which were also handled on the same wet-market have come into focus as potential reservoir hosts. Sequences of ancestral SARS-like-CoV

have been detected in bats, but so far – despite several attempts – no coronaviruses have been isolated from *Rhinolophus* bats (Drexler et al., 2010; Falcón et al., 2011; Lau et al., 2005; Li et al., 2005; Shi and Hu, 2008; Yang et al., 2013).

Although a plethora of diverse coronaviruses of distinct groups have been detected in various bat species via molecular-biological techniques, it has still not finally been proven that bats are the natural reservoir host of the SARS-CoV (De Souza Luna et al., 2007; Gloza-Rausch et al., 2008; Lau et al., 2013; Pfefferle et al., 2009; Poon and Peiris, 2008; Tao et al., 2012; Tsuda et al., 2012; Yang et al., 2013).

MERS Corona Virus

In September 2012, The World Health Organization (WHO) reported on human cases of infectious pneumonia caused by a novel coronavirus (Middle East Respiratory Syndrome virus; MERS-CoV[2]) in Eastern Saudi Arabia (Buchholz et al., 2013; Guberina et al., 2013; Mailles et al.,2013). Similar to SARS, the disease is caused by a betacoronavirus and transmissible from human to human. By August 2013, 93 human infections were confirmed and a case–fatality rate of 68 percent was reported, ranging from the Middle East (Saudi Arabia, Jordan and Tunisia) to Europe (England, France and Germany) (Zaki et al., 2012).

Phylogenetically, MERS-CoV is related to viruses circulating in European bat populations (Cotten et al., 2013). The closest European relative is P.pipi/VM314/2008/NLD (87.7 percent) detected in *Pipistrellus pipistrellus* in the Netherlands, followed by H.sav/J/Spain/2007 (*Hypsugo savii,* Spain) and E.isa/M/Spain/2007 (*Eptesicus isabellinus*, Spain) (13.2) (Falcón et al., 2011; Reusken et al., 2010). A study screening Ghanaian and European bats obtained MERS-related viruses from *Nycteris gambiensis* in Ghana and more European Pipistrelle bats (*Pipistrellus nathusii, Pipistrellus pipistrellus, Pipistrellus pygmaeus*) (Annan et al., 2013). More recently, an even more closely related bat coronavirus was identified in South Africa (*Neoromicia zuluensis)*, distinct by solely a single amino acid change in the translated 816 nt RdRp gene (Ithete et al., 2013). Eventually the MERS-CoV was detected in Saudi Arabian bats (*Taphozous perforates*) (Memish et al., 2013). Still, only hints are suggesting bats as natural reservoir hosts, yet solid evidence is missing. The question as to whether bats could act as vectors, or whether the virus might require amplification hosts (e.g. camels) is presently subject of further studies (Reusken et al., 2013).

[2] Initially named as CoV-EMC and CoV-London 2.

1.2.2.2 Filoviruses

Members of the *Filoviridae* are divided into two genera - Marburg-like and Ebola-like - viruses. Both are belonging, together with rhabdoviruses, bornaviruses and paramyxoviruses, to the order *Mononegavirales.* Experimental and natural infections with filoviruses do not appear to be associated with consecutive disease developments in bats (Leroy et al., 2005; Swanepoel et al., 1996; Towner et al., 2009).

Marburg virus

Late in the summer of 1967 the first member of the family *Filoviridae* was identified and isolated in Marburg, Germany (Siegert et al., 1968; Slenczka 2007). A severe disease of unknown etiology was contracted by 32 persons in Germany and the former Yugoslavia, of whom seven eventually died (5/24 in Marburg, 2/6 in Frankfurt, 0/2 in Belgrade) (Martini and Siegert, 1971). The patients revealed flu-like and gastro-intestinal symptoms. Later on, 25 percent developed signs of hemorrhagic diathesis and bled from all body orifices and needle punctures. The developments of severe hemorrhagic signs were a *signum mali ominis*, as all patients with fatal courses developed hemorrhaging (Slenczka, 2007).

It quickly became apparent that all initially infected patients from Marburg, Frankfurt and Belgrade were employees of research or industrial facilities focusing on sera and vaccines (Behringwerke, Marburg; Paul Ehrlich Institute, Langen; Institute Torlack, Belgrade). Furthermore, all of them had been in direct contact with clinical specimens of vervet monkeys (*Cercopithecus aethiops*) imported from Lake Victoria, Uganda (Siegert et al., 1968). All animals seemed to be in good health upon arrival; only in Belgrade the animals were kept over a longer period, and mass mortality (33 percent) was observed later on (Slenczka and Klenk, 1999). Three months after the initial human case, the virus was successfully isolated, visualized via negative staining electron microscopy and consecutively named Marburg virus Lake Victoria (MARV) (Siegert et al., 1968).

In consecutive experimental MARV infection studies, vervet monkeys showed clinical symptoms and died, leading to the assumption that they were not acting as the natural MARV reservoir hosts (Peterson et al., 2004). In 2007, two studies described antibodies reactive to MARV in bat sera and specific RNA detection (*Rousettus aegyptiacus, Rhinolophus eloquens* and *Miniopterus inflatus*) in samples from Gabon and the Democratic Republic of Congo (DRC) (Swanepoel et al., 2007; Towner et al., 2007). Two years later, a sero-prevalence study demonstrated the circulation of MARV and EBOV in Gabonese bats, shortly after MARV was eventually isolated from *Rousettus aegyptiacus* and the bat reservoir hypothesis was proved correct (Pourrut et al., 2009; Towner et al., 2009).

Consecutive cases of MARV infections in humans were sporadically connected to mine-working or tourist visits of mines inhabited by bats (Adjemian et al., 2011; Amman et al., 2012; Brauburger et al., 2012; Chen and Lercher, 2009; Fujita, 2010; Timen et al., 2009).

Ebola virus

The genus of *Ebola-like viruses* comprises five distinct species, four of which cause severe hemorrhagic fever similar to MARV with case–fatality rates between 41 percent (Sudan Ebola virus; SEBOV) and 89 percent (Zaïre Ebola virus; ZEBOV) in humans and primates (Formenty et al., 2003; Onyango et al., 2007). With the exception of Reston Ebola Virus (REBOV[3]), all Ebola-like viruses are endemic in Africa and capable of inducing severe diseases in primates and humans (Negredo et al., 2011). Ebola virus first emerged in Zaïre (nowadays Democratic Republic of Congo; DRC) in 1976 and simultaneously in Sudan and was named after the Congolese Ebola river (Emond et al., 1977). During the search for the reservoir host, different animals have been suspected and examined (Peterson et al., 2004). Finally, 30 years later, studies reported first evidence that bats (*Rousettus aegyptiacus, Hypsignathus monstrosus*, and *Epomops spp.*) were possible reservoir hosts of Ebola-like viruses (Biek et al., 2006; Leroy et al., 2005; Pourrut et al., 2009; Swanepoel et al., 1996). An outbreak in DRC described bats as the vectors responsible for this occurrence: During their annual migration, fruit bats settled for a few weeks in fruit trees in Luebo (DRC). Shortly afterwards, a massive ZEBOV outbreak horrified the region. 260 humans became infected and 186 eventually died (Leroy et al., 2009).

Overall, there are strong hints but as yet no proof or evidence to support the reservoir host hypothesis. Whether bats are the natural reservoir hosts of Ebola-like viruses has yet to be determined.

1.2.2.3 Henipaviruses

Henipavirus is a genus within the family *Paramyxovirinae* in the order *Mononegavirales*. So far, they comprise three viruses: Hendra virus, Nipah virus and the recently discovered Cedar virus (CeV) (Marsh et al., 2012). While closely related to Hendra virus and Nipah virus, CeV shows distinct features like pathogenicity and virulence in ferrets and has never been reported to spill over to the human population. Beside these three viruses, antibodies against Henipa-like viruses were described in bat sera in Africa and South-East Asia as well as the detection of related nucleic acids in African, Indian, American, Australian and European bats (Bossart et al., 2007; Drexler et al., 2009, 2012a; Epstein et al., 2008; Hayman et al., 2008; Li et al., 2008; Weiss

[3] REBOV is endemic in the Philippines and described as causing non-lethal sub-clinical infections in humans (Miranda et al., 1999).

et al., 2012a). But, as only Hendra virus and Nipah virus are reported to have any human-pathogenic significance, this chapter is limited to these two viruses.

Hendra virus

In 1994, horses fell sick on a property in Brisbane's suburb Hendra in Australia, suffering from a respiratory disease (Murray et al., 1995). Shortly after the horses died the trainer and the properties' stable hand became ill. Six days later the trainer died and the stable hand started to recover gradually. All infections were caused by the same paramyxovirus, named after the place of its first occurrence: Hendra virus. The search for the animal reservoir revealed many hints towards bats (genus *Pteropus*) before Hendra virus was directly isolated from fruit bat urine in 2000 (Halpin et al., 2000). So far, 78 horses, 1 dog and 7 humans got infected, with a case–fatality rate of 58 percent in humans (Smith and Wang, 2013). Still, the actual transmission route between bats, horses and humans has not been identified yet. More recently, a dog was evidently infected on one of the properties and developed high antibody levels (Mahalingam et al., 2012; Mendez et al., 2012). Whether dogs or the Australian paralysis tick, as was assumed in another study (Barker, 2003), represent an alternative missing link in Hendra virus transmission is not yet determined.

Nipah virus

In 1995, Nipah virus emerged, closely related to Hendra virus, with an even higher case–fatality rate (Chua et al., 2000). Similar to the cases of Hendra virus infection, bats were identified as potential reservoir hosts (Yob et al., 2001). And like Hendra virus, Nipah virus required an amplification host between bat and human at its first emergence (Paton et al., 1999). In this case swine acted as interim hosts instead of horses, before eventually Nipah virus became transmissible from bats to humans and human to human, respectively (Chadha et al., 2006; Gurley et al., 2007). So far, Nipah virus outbreaks have occurred in Malaysia, Cambodia, Singapore and India and are endemic in Bangladesh.

1.2.2.4 Lyssaviruses

Lyssavirus, a genus within the family *Rhabdoviridae*, are the best-studied viruses of bats comprising 15 virus species: Rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus types 1 and 2 (EBLV-1 and -2), Australian bat lyssavirus (ABLV), Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV), West Caucasian bat virus (WCBV), Shimoni bat virus (SHIBV), Bokeloh bat lyssavirus (BBLV), Ikoma lyssavirus (IKOV) and Lleida bat virus (LLBEV) (Aréchiga Ceballos et al., 2013). Bats are natural reservoirs for 13 out of these 15 viruses; only MOKV and IKOV have not been detected in bats so far.

The Rabies virus is the prototype of the genus and is causing a fatal neurological paralytic disease in humans with case–fatality rates of virtually 100 percent. Only one case is reported where a 15-year-old girl survived the successive development of clinical rabies disease, one month after she was bitten by a bat, by inducing a coma and treatment with ketamine, midazolam, ribavirin and amantadine (Willoughby et al., 2005). However, another study describes the failure of this treatment in a clinical rabies infection caused by DUVV (Van Thiel et al., 2009).

RABV is circulating in the New World bat populations of the Americas, whereas the other members of the genus *Lyssavirus* are circulating in Africa (LBV, MOKV, DUVV, SHIBV, IKOV), Asia (ARAV, KHUV, IRKV, WCBV, LLEBV) and in Australia which is considered to be Rabies-free (ABLV) and Europe (EBLV-1 and -2, BBLV) (Kuzmin et al., 2011; Okonko et al., 2010).

By now, lyssaviruses have been assigned to seven different genotypes, all except for genotype 2 reported to cause clinical rabies disease in humans: Genotype 1: (RABV), genotype 2: (LBV), genotype 3: (MOKV), genotype 4: (DUVV), genotypes 5 und 6: (EBLV 1, 2), genotype 7: (ABLV). After all, clinical rabies is a preventable disease. Available vaccination protects from all genotypes except for genotype 2 (Badrane et al., 2001), while it needs to be taken into account that many of the novel lyssaviruses have not yet been assigned to genotypes, and hence the protection against these viruses is unknown.

Beside the vast distribution of bats and the high prevalence and diversity of lyssaviruses, only three spill-over events from bat to humans have been reported in Europe (Johnson et al., 2010).

1.2.3 Viruses in European Bats

Viruses that have been detected in European bats are listed in Table A2: Viruses Found in European Bats. Selected viruses are described in the following section. Moreover, the correlation of all bat viruses with the bat host species is displayed in Figure 4.

1.2.3.1 Adenoviridae

The first isolation of an adenovirus (Bt AdV-2) from European bats (*Pipistrellus pipistrellus*) was reported in 2009 (Sonntag et al., 2009). As part of this thesis, qPCR screening of additional 330 bats of different species was performed. The whole genome was sequenced, annotated and phylogenetically analyzed. The results are displayed and discussed in Manuscript I.

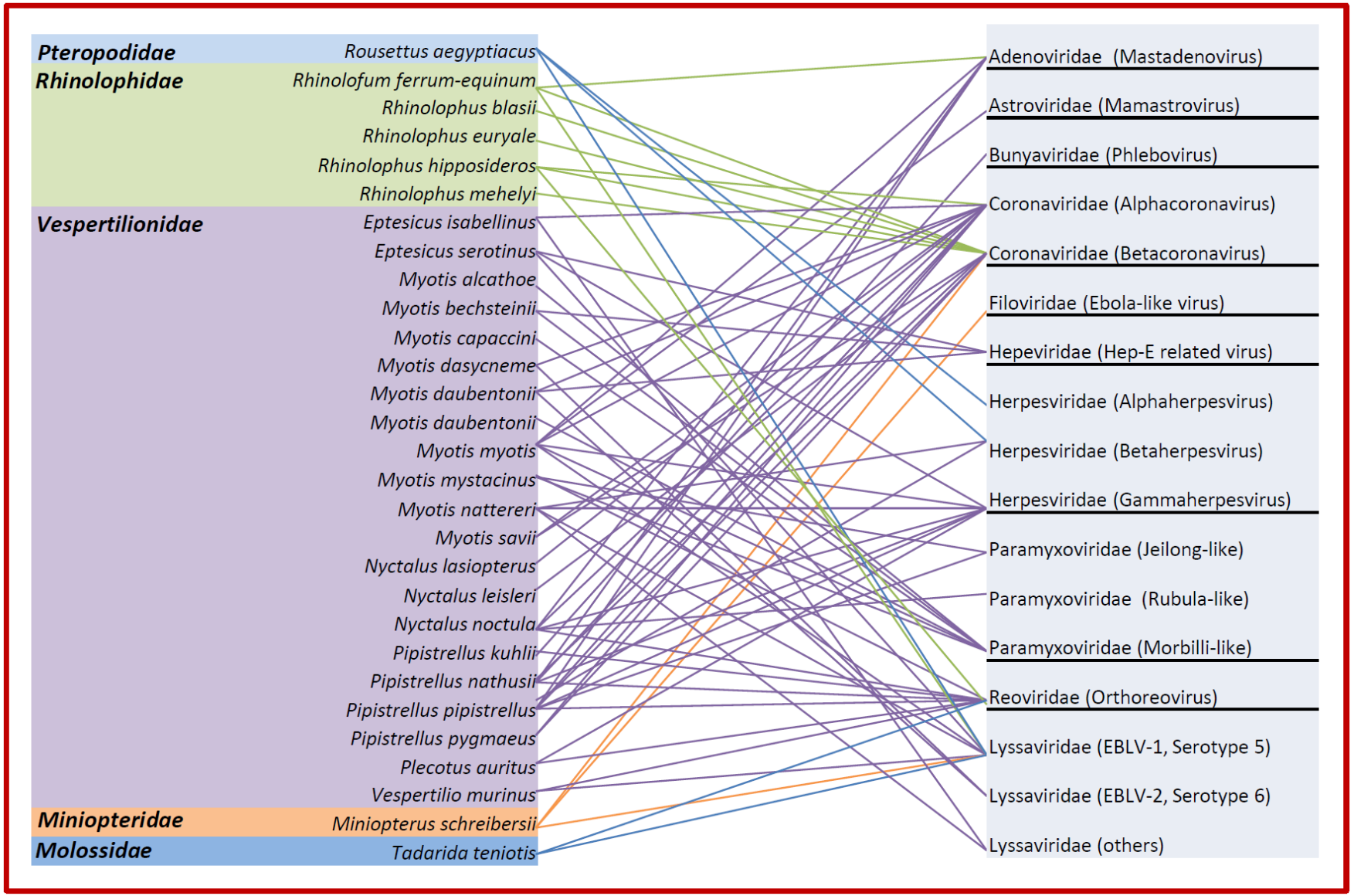

Figure 4 Bat species correlated to viral species detected in European bats.

Subsequently, the establishment of a novel species was proposed which was accepted by the International Committee of Virus Taxonomy (ICTV) (The proposal is given in Manuscript II).

In 2010, Jánoska et al. reported the detection of two novel mastadenoviruses in Hungarian bats (Jánoska et al., 2011). HUN/2009-Josvafo2009 was detected in two Greater Horseshoe bats (*Rhinolophus ferrum-equinum*), and HUN/2007-batAdVnt was detected in the Common Noctule bat (*Nyctalus Noctula*).

Another mastadenovirus was detected by PCR from fecal specimens of Greater Mouse-eared bats (*Myotis myotis*) roosting in a cave in Germany (Drexler et al., 2011).

1.2.3.2 Astroviridae

Six novel astroviruses belonging to the genus *Mamastrovirus* were described after detection in fecal specimens of Greater Mouse-eared bats (*Myotis myotis*) collected in three consecutive years in a roosting cave in Germany (Drexler et al., 2011).

1.2.3.3 Bunyaviridae

Toscana virus was isolated from the brain of a Kuhl's pipistelle (*Pipistrellus kuhli*) in Italy in 1988 (Verani et al., 1988). The Toscana virus belongs to the genus *Phlebovirus* and is usually transmitted via sandflies.

1.2.3.4 Coronaviridae

A variety of 30 novel alphacoronavirus sequences were obtained from northern German pond bats (*Myotis dasycneme*/19), Nathusius' bats (*Pipistrellus nathusii*/2), Soprano pipistrelles (*Pipistrellus pygmaeus*/3) and Daubenton's bats (*Myotis daubentonii*/6) (Gloza-Rausch et al., 2008). The novel sequences were forming four distinct monophyletic lineages within the alphacoronaviruses. In a study by Drexler et al. in 2011 adeno- and astroviruses were also described, and a novel alphacoronavirus Bat CoV N78/5 was detected simultaneously in Greater Mouse-eared bats (*Myotis myotis*) in Germany (Drexler et al., 2011).

14 Novel alpha- and betacoronaviruses were detected by PCR in Spain (Falcón et al., 2011). Thereof, 12 novel alphacoronaviruses were described in *Miniopterus schreibersii*/1, *Myotis blythii*/1, *Pipistrellus spp.*/1, *Myotis daubentonii*/1, *Hypsugo savii*/1, *Nyctalus lasiopterus*/5, *Myotis myotis*/1, *Pipistrellus kuhlii*/1 and two novel betacoronaviruses in *Hypsugo savii*/1 and *Eptesicus isabellinus*/1. Additional 18 alpha- and betacoronaviruses were described in bats in the Netherlands. Alphacoronaviruses were found in four bats species (*Nyctalus noctula*/3), (*Pipistrellus pipistrellus*/1), (*Myotis dasycneme*/9), (*Myotis daubentonii*/3) and one betacoronavirus in *Pipistrellus pipistrellus* (Reusken et al., 2010).

Betacoronaviruses comprise the human-pathogenic CoV-229E, CoV-NL63 and the SARS-CoV. 33 different betacoronaviruses have been detected by PCR in Bulgarian bats [(*Miniopterus schreibersii*/9), (*Nyctalus leisleri*/1), (*Rhinolophus blasii*/10), (*Rhinolophus*

ferrum-equium/4), (*Rhinolophus Euryale*/7) and (*Rhinolophus meheli*/2)] (Drexler et al., 2010), 16 of which appeared to be related to SARS-CoV, and consecutively named SARS-like-CoV (*Rhinolophus eurale*/6), (*Rhinolophus blasii*/5), (*Rhinolophus ferrum-equium*/3), (*Rhinolophus meheli*/2). In Slovenia, 14 coronaviruses have been detected in *Rhinolophus hipposideros*, all of which are very closely related to each other and overall constitute a sister-clade to the SARS-like-CoV (Rihtarič et al., 2010). Another study conducted on German, Ukrainian and Romanian bats revealed novel betacoronaviruses in *Pipistrellus pipistrellus*, *Pipistrellus nathusii* and *Pipistrellus pygmaeus* (Annan et al., 2013).

1.2.3.5 Filoviridae

In 2002, the first reported outbreak of an Ebola-like virus, named Lloviu virus (LLOV), in an European bat population occurred in France, Spain and Portugal (Negredo et al., 2011). Several colonies of Schreiber's bats (*Miniopterus schreibersii*) suddenly declined due to an unknown disease. LLOV was found in animals that showed signs of viral infection, but not in healthy bats co-roosting in the caves (*Myotis myotis*). The lack of successful isolation of LLOV prohibits the experimental infection of Schreiber's bats to clarify whether LLOV is the first Ebola-like virus capable of inducing virulence in bats. This would challenge the hypothesis of bats as potential reservoir hosts for Ebola-like viruses.

Schreiber's bats are distributed in distinct lineages throughout Oceania, Africa, Southern Europe and South-East Asia (Appleton et al., 2004) (Table A1: Characteristics of European Bat species). LLOV is related to Ebola-like viruses that can be found in Africa. Schreiber's bats are discussed to transmit and maintain the virus across different lineages throughout their habitats, although no studies are available to prove this hypothesis.

1.2.3.6 Hepeviridae

Hepevirus-related sequences have been obtained by PCR from fecal specimens of German bats (*Eptesicus serotinus*/1) and Bulgarian bats (*Myotis bechsteinii*/1), (*Myotis daubentonii*/2) (Drexler et al., 2012b).

1.2.3.7 Herpesviridae

Seven distinct gammaherpesviruses (in *Eptesicus serotinus, Myotis nattereri, Pipistrellus nathusii, Pipistrellus pipistrellus, Myotis myotis, Nyctalus noctula* and *Plecotus auritus*) and one novel betaherpesvirus *(*in *Myotis nattereri* and *Pipistrellus pipistrellus*) have been discovered in Germany by PCR screening (Wibbelt et al., 2007). Subsequently, a novel gammaherpesvirus was described, obtained from tissues of a moribund bat (*Eptesicus serotinus*) in Hungary (Molnár et al., 2008). In 2011, Jánoska et al. described a novel alpha- and betaherpesvirus in Hungary, both obtained from one captive *Rousettus aegyptiacus* fruit bat by PCR (Jánoska et al., 2011).

1.2.3.8 Paramyxoviridae

During a PCR screening for unknown viruses conducted in this thesis, three distinct paramyxoviruses were detected in German bats. The results are displayed and discussed in Manuscript III.

Another study published in the same year described another 12 different paramyxoviruses in bats from Germany (*Myotis bechsteinii, Myotis daubentonii, Myotis myotis* and *Myotis mystacinus*) and Bulgaria (*Myotis alcathoe* and *Myotis capaccini*), all of them related to the genus *Morbillivirus* (Drexler et al., 2012a).

1.2.3.9 Reoviridae

During this thesis three novel orthoreoviruses were isolated and characterized. The results are displayed and discussed in Manuscript IV.

At the same time, a group in Italy detected further 19 orthoreoviruses of the same genus (*Myotis kuhlii*/15, *Rhinolophus hyposiderus*/1, *Tadarida teniotis*/2 and *Vespertilio murinus*/1) (Lelli et al., 2013).

1.2.3.10 Rhabdoviridae

In Europe six distinct lyssaviruses (EBLV-1, EBLV-2, Bokeloh virus, Lleida bat lyssavirus, West Caucasian bat lyssavirus and Dimarrhabdovirus) circulate in the bat populations. They have been detected in numerous species: EBLV-1 was detected throughout Europe in bats of the species *Eptesicus serotinus* and *Vespertilio murinus* (Bourhy et al., 2005; Fooks et al., 2003; Schatz et al., 2013; Van Der Poel et al., 2005). Moreover, EBLV-1 was detected in Spanish *Myotis myotis, Myotis nattererii, Miniopterus schreibersii, Rhinolophus ferrum-equinum* and *Tadarida teniotis* (Serra-Cobo et al., 2002) as well as in one *Rousettus aegyptiacus* in a zoo in the Netherlands (Bourhy et al., 1992). EBLV-2 has been described in *Myotis daubentonii* and *M. dasycneme* throughout Europe (Brookes et al., 2005; Fooks et al., 2003; Johnson et al., 2003; Serra-Cobo et al., 2002). More recently, novel bat lyssaviruses were described in Spain (Dimarrhabdovirus in *Eptesicus isabellinus* and Lleida bat lyssavirus in *Miniopterus schreibersii*) and France (Bokeloh bat lyssavirus in *Myotis natteri*) (Aréchiga Ceballos et al., 2013; Picard-Meyer et al., 2013; Smyth et al., 1999; Vázquez-Morón et al., 2008). West Caucasian bat lyssavirus was isolated just once from *Miniopterus schreibersii* on the European side of the Caucasian mountains (Kuzmin et al., 2005).

1.3 VIRUS DETECTION AND CHARACTERIZATION METHODS

1.3.1 Virus Detection

When screening for known and novel viruses, certain strategies are widely used, based on the fact that all viruses have three things in common that can be utilized for virus detection: First they are infectious particles, secondly they consist of proteins, and finally they encode for these proteins with their own genomes.

1.3.1.1 Detection Based on Infectious Particles

Successful virus detection based on infectivity is constrained by the sample quality. Intact and infectious viral particles are a prerequisite for success, and adequate sample storage is a difficult task. When using infectivity as a detection parameter, a susceptible host organism is indispensable. Furthermore, the virus infection necessarily needs to result in a clearly recognizable alteration in the host organism. The obvious advantage of this approach is that, if successful, it provides a full virus isolate which can be the basis for further virus characterization studies.

In theory, animals can be used to screen for novel viruses (*in vivo*), but for ethical and economic reasons this is a very rare option. Nevertheless, suckling mice were widely used for the cultivation of novel viruses several decades ago (McIntosh et al., 1967; Ramig, 1988). Also, the infection of embryonated chicken eggs (*in ovo*), though an older technique, is still frequently used as a valuable tool (Fulton and Isaacs, 1953; Ramp et al., 2012). A successful infection of the embryo can easily be recognized visually by candling the eggs, since an infected embryo often appears atrophied compared to uninfected ones. In addition, the presence of viruses or bacteria in the egg's allantoic fluids can be determined by a quick and easy hemagglutination assay (HA) (Chu, 1948). Though only a limited number of viruses are capable of inducing viral replication in eggs, they can serve as complementary detection methods for novel viruses. However, the gold standard is virus cultivation in cell culture (*in vitro*) (Leland and Ginocchio, 2007). Similar to the virus–host tropism that can be observed *in vivo*, cell cultures often mimic this *in vitro* through a distinct susceptibility for only certain virus types. As it is usually not predictable which viruses to expect, cell lines are usually chosen according to their degree of universality.

Another method harnessing the infectious particle character and the structure of viral proteins is transmission electron microscopy (TEM). Using infectious and thus intact virus particles for TEM facilitates effective virus identification through visualization of viruses, hence typical structures and particle size are easier to recognize. TEM offers the "open view" into the sample, provided that it is sufficiently clean and concentrated (Biel and Gelderblom, 1999).

Even though the detection limit for TEM (10^5 virus particles/ml) is not always reachable (Laue and Bannert, 2010), TEM is a powerful method for the rapid identification of pathogens from clinical specimens, environmental samples and infected cell cultures.

1.3.1.2 Virus Protein-Based Detection

In general, virus detection (beside spectroscopy) through viral proteins requires coupling to a detection molecule (Clark and Adams, 1977). As detection molecules either specific aptamers are utilized or, more commonly, specific antibodies (Iqbal et al., 2000; Zhang et al., 2004). Antibodies are mostly labeled with fluorochromes and thus become detectable. A detection based on virus protein–antibody interaction works in two directions: detection of viral particles by detection molecules or detection of antibodies by viral particles. In the first case, the required detection molecule consists of labeled antibodies capable of binding specifically to a virus protein, whereas in the second case specific viral proteins are immobilized to bind reactive antibodies in the sample. This is followed by a second set of antibodies acting as detection molecules which are directed towards the host species (e.g. anti-bat) to detect all bound antibodies in the sample. Thus, virus protein-based detection is limited to the detection of known pathogens. Prior knowledge of the expectable pathogen serogroup would be necessary to detect known or novel viruses, as a screening for all known virus serogroups is not feasible. The detection of novel viruses utilizes the cross-reactivity of serotypes within a virus serogroup. Though protein-based detection methods are a powerful tool, their suitability is restricted when unbiased virus detection is desired.

1.3.1.3 Virus Genome-Based Detection

All viruses are encoded by their distinct genomes. They are taxonomically divided based on encoding of their own genomes either in DNA or RNA, and are subsequently distinguishable by their double-stranded (ds) and single-stranded (ss) genome structure. The RNA viruses with ss-genomes are either positive-strand or negative-strand orientated, and the architecture of the genome is indicated by the terms segmented, linear or circular (Figure 5) (Tidona and Darai, 2001).

To detect viruses based on their genome information, the Polymerase Chain Reaction (PCR) is the method of choice, because it is sensitive, highly adaptable, cheap and fast (Belák and Ballagi-Pordány, 1993; Elnifro et al., 2000; Mackay, 2002). PCR is based on DNA; therefore, the detection of RNA viruses requires the prior transcription into complementary DNA (cDNA). Different PCR approaches are used for virus detection, offering specific answers to a variety of research questions (e.g. conventional PCR, nested PCR, hemi-nested PCR, generic PCR, qPCR, inversePCR, multiplexPCR, RACE PCR and random PCR).

Their specificity for the genomic target varies from high (qPCR) to unbiased (randomPCR), depending on the amount of degenerated nucleotides in the primer sequence, annealing temperature and conservation of the target sequence (Compton, 1990). The higher the degree of unbiased amplification, the more effort is necessary to obtain a specific sequencing result. While a specific conventional PCR results in a single amplicon that contains the target sequence (e.g. Ebola virus), a generic PCR assay with a higher level of degradency for amplifying different sequences (e.g. all *Filoviridae*) may result in multiple amplicons. Here a gel extraction and often cloning into vectors is necessary to obtain single sequences. In contrast, the randomPCR, with unbiased amplification, results not only in multiple bands, but in a smear of different sequences that need to be scattered for sequencing (Ambrose and Clewley, 2006). This scattering can be achieved by cloning into sequencing vectors with consecutive Sanger-sequencing or by *Next Generation Sequencing* (NGS).

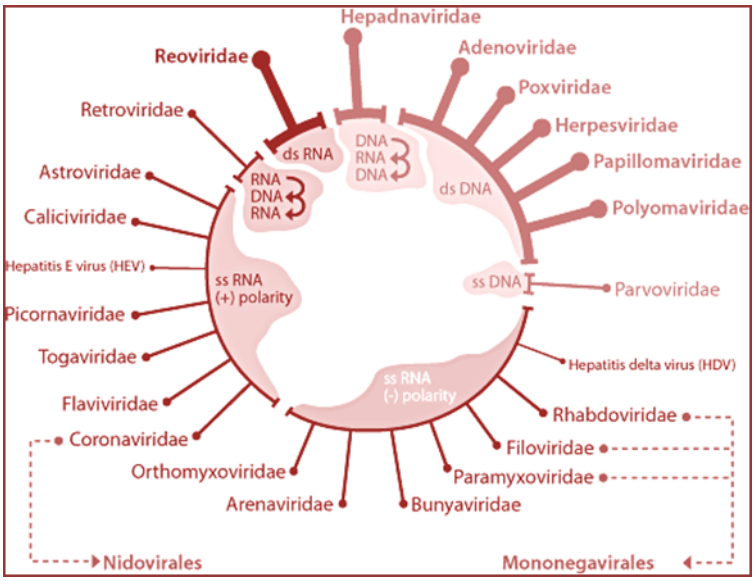

Figure 5 Overview on genome characteristics and orientation of main virus families. Original diagram from Antiviral InteliStrat Inc. (www.antiviralintelistrat.com), all rights reserved. Reproduced with the authors' permission.

1.3.2 Next Generation Sequencing

Next Generation Sequencing (NGS) is revolutionizing the world of molecular biology and virus detection. Overall, NGS comprises high-throughput approaches that generate sequence information to an amount which is not feasible to fully analyze by conventional techniques (e.g. manual BLAST). An increasing number of different technologies are available, all differing in the amount of sequence data generated and the output read length (Radford et al., 2012). They have in common that theoretically all nucleic acids in the sample are sequenced unbiased and simultaneously.

1.3.2.1 Sequencing Platforms

Here the techniques used in this study are briefly described (Ansorge, 2009; Lipkin, 2009; Metzker, 2010; Radford et al., 2012). An overview of available NGS methods, basic technologies and their output is given in Table 1.

454 Sequencing (Roche Diagnostics)

Starting with blunt-end dsDNA as a sample, adaptors are ligated on both ends before the whole construct is coupled onto micro beads (Margulies et al., 2005). Subsequently, a water-in-oil emulsion is generated, with a sophisticated mixture, creating high probabilities to obtain only a single micro bead with associated reagents per droplet. Thermal cycling of the emulsion results in simultaneous PCR amplification in all droplets. Afterwards, the emulsion is broken and the amplicons are concentrated and scattered into the wells of a pico titre plate. Again, with high probability only one bead per well is present in the pico titre plate. The actual sequencing reaction is performed by pyrosequencing (PSQ). The advantage of 454 sequencing is the read length of approximately 500 bases, the disadvantage is the error rate in individual reads of up to 1% (insertions and deletions) due to e.g. homopolymers.

Ion Torrent (Life Technologies)

The differences between 454 sequencing and Ion Torrent mainly begin at the step when scattering of the sequences coming from the broken emulsion is performed. In contrast to 454 sequencing, the sequences are loaded onto an ion-sensitive semiconductor. The sequence signal here is detected via a change in pH instead of measuring fluorescence (454 PSQ). The data output of Ion Torrent exceeds the 454 sequencing output, though it is more cost-effective. In contrast, 454 sequencing is more expensive but on average delivers reads of higher quality.

Table 1 Overview on current NGS technologies

Sequencing method	Adapters	Amplification	Separation of sequences	Sequencing	Approx. read length (nt)	Read output (maximum)
Roche 454*	Adapters	Emulsion PCR	Micro beads and pico titre plate	Pyrosequencing	400-800	0.8 Mio
SOLiD	Adapters	Emulsion PCR	Beads on glass slide	Ligation	50-75	1,400 Mio
Illumina*	Adapters	Bridge amplification *in situ*	Glass slide hybridization	Reversible terminators	25-500	300-1,500 Mio
Helicos	Poly(A) adapter	No amplification	Flow-cell hybridization	Reversible terminators	25-55	35 Mio
PacBio	Hairpin adapter	Linear amplification	Captured by DNA polymerase in microcell	Fluorescence labeled dNTPs	1,000	n/a
Ion Torrent	Adapters	Emulsion PCR	Ion spheres and high density array	Detection of released H^+	35-400	5 Mio

*These technologies are available with different scales of throughput.
Table adapted from (Radford et al., 2012).

1.3.2.2 Sequencing Approaches

There is a variety of sequencing goals that can be reached by different sequencing technologies (Table 1). The preferable approach depends mainly on the actual research question and available sequencing platforms.

Whole Genome Sequencing

Whole genome sequencing (WGS) is the classical purpose of high-throughput sequencing, aiming to obtain the complete genome of an organism. Therefore, the genomic DNA in one sample is sheared (e.g. enzymatically or ultra-sonically) before all fragments are sequenced simultaneously. Consecutively, the single sequences obtained are assembled either *de novo* by sequence overlaps between the reads or by mapping onto genome scaffolds. The latter requires knowledge about the genome in advance. For instance, for the purpose of sequencing the whole genome of a novel orthoreovirus known and related orthoreoviruses can be used as a scaffold. When mapping to a scaffold, the read-length is not crucial. In contrast, the *de-novo* assembly works without any prior knowledge of the sequence, therefore longer reads are beneficial to increase the chance of longer overlapping regions.

Amplicon Sequencing

As an example of amplicon sequencing, the whole bacterial diversity in a stool sample can be obtained (Tamaki et al., 2011). All bacteria share the highly conserved 16S rRNA region and thus can be amplified with specific 16S primers. In a stool sample this may result in millions of amplified 16S sequences, many of them originating from different bacteria. To sequence all single 16S sequences, larger reads are preferable in order to span the whole amplicon. Furthermore, error probabilities should be minimal, as single nucleotide polymorphisms (SNPs) shall get detected in the highly conserved regions. Deep sequencing (7-fold sequence coverage) or ultra-deep sequencing (>7-fold coverage of the sequence) contribute to increased reliability.

Metagenomics

Metagenome sequencing starts from an unknown sample composition which might contain a population of differing life forms. If a sufficient amount of sample material is available, the direct extract can be used, but if the material is limited amplification is necessary. In contrast to amplicon sequencing, the sample cannot get amplified by a specific simple primer set, thus random amplification is necessary. This sequencing approach results in a plethora of different sequences obtained from differing sources (i.e. viruses, bacteria, host genome) (Bexfield and Kellam, 2011). For data analysis all sequences need to be compared, e.g. to the GenBank database.

Nowadays, various metagenomic NGS approaches already provide reliable solutions for pure and concentrated viruses (e.g. from cell culture), but when it comes to clinical specimens like blood, fluids or even infected organ tissue the successful detection of viruses is less likely. In cell-culture systems viruses propagate to higher virus titers than in native host organisms. On the other hand, using tissue for virus detection allows for the elucidation of viral infections directly at the place of viral replication. This in turn allows for the instant correlation of physiological host effects (*phenotype*) with the causing viral agent (*genotype*). Other clinical specimens are mainly host excretions (e.g. urine, blood, fluids) which are dependent on transportation fluids and therefore less concentrated. In contrast, the amounts of viruses in organ tissue are more stable because the availability of viruses in infected organ tissue is less dependent on stages and cycles of replication, viremia and shedding, respectively. Although the detection of viruses directly from infected organ tissue provides obvious and valuable advantages, only few studies have used this approach. Detection of viruses is difficult because an extensive supply of host nucleic acids competes with the targeted virus nucleic acids. Reliable virus purification from tissue remains a challenge.

The first step of purification is the disruption of the tissue to release viral particles. Simultaneously, host nucleic acids, proteins and cell organelles are also released. A strategy is necessary for enriching the viral particles while decreasing the host genome. Following purification, the viral nucleic acids need to be amplified to increase the likelihood of detection. For the unbiased detection of viruses, random primers are the method of choice as no prior knowledge of the target sequence is needed. The advantage of this technique is also its disadvantage: Every sequence present in the sample will be amplified simultaneously. Here one needs to take into consideration that the amount of host nucleic acids far exceeds the amount of virus nucleic acids per cell. Every single mammalian cell contains the full host genome (e.g. human genome: about 3 Gb) and numerous amounts of different RNA species.

1.3.3 Sequence Analysis *in silico*

In section 1.3.1 possibilities of virus detection were described. Once, a virus is detected the resulting sequence will be analyzed. This section focuses on the subsequent *in silico* characterization of novel viral pathogens.

1.3.3.1 Annotation of Viral Genomes

After a novel virus genome has been sequenced, the next step is the annotation and prediction of proteins within the raw sequence. To predict possible proteins, open reading frames (ORF) have to be identified. Virtually all eukaryotic mRNAs start with the initiation codon AUG encoding for the amino acid methionine (Met) (Nirenberg et al., 1965). Prokaryotic

initiation is also encoded by Met but via an aberrant codon usage, more often using GUG and UUG beside AUG (Cavener and Ray, 1991; Kozak, 1983). The end of the mRNA is defined by one out of three stop codons. These are the three possible base triplets that do not encode for any amino acid. Hence no respective tRNA is existent and the transcription will be terminated (UAA, UAG and UGA) (Nirenberg et al., 1965). The length between start and stop codon is called ORF which encodes for the precursor mRNA. Some viruses process the precursor mRNA, similar to eukaryotes, into differing mature mRNAs by alternative splicing. Alternative splicing was discovered on adenoviruses, and the mechanism is regulated by a complex composition of factors (e.g. cisRNA and the secondary structure of the RNA) (Chow et al., 1977; Wang and Burge, 2008). Upon transcription of the mRNA some proteins undergo further protein splicing (enzymatic cleavage of protein inteins or peptide fusion). Splicing junctions are here mainly cysteine and serine (Pietrokovski, 1998).

The annotation of novel viruses is often performed according to proteins of already known related viruses. Nowadays, software is available facilitating protein annotation. For instance, the *Gene Locator and Interpolated Markov ModelER* (GLIMMER) (Salzberg et al., 1998) or *FGENESV* (www.softberry.com) are options tailored to viral genomes. In addition to the prediction of secondary protein structures (α-helix, β-sheet, turns and coils; e.g. by EMBOSS: http://emboss.sourceforge.net), the annotation of viral genomes allows for a comparison between virus isolates on a molecular level and may lead to first hints regarding different functionalities.

1.3.3.2 Phylogenetic Sequence Analysis

Molecular Evolution

The goal of a phylogenetic sequence analysis is to discover how life forms are related to each other and how they diverted through evolution over time. The foundation is the genome information every living cell and every virus carries in form of either RNA or DNA (Lemey et al., 2010).

The genetic information is a triplet code of the four nucleotides encoding for 20 proteinogenic amino acids (aa) by 4^3 (=64) possible codons (61 sense and three stop codons). Occasionally, non-complementary nucleic acids become incorporated during reproduction of the genetic information (von Haeseler and Liebers, 2003). The genetic information is from then on altered, resulting in so-called point mutations. They are classified as *transitions* if either a purine (A, G) is replaced by a purine, or a pyrimidine (C, T) is replaced by a pyrimidine. When a purine is replaced by a pyrimidine and vice versa they are classified as *transversions*. Transitions and transversions not resulting in aa changes are called silent mutations, while mutations resulting in an aa change are called non-silent mutations. Additionally, insertions

and deletions of nucleotides can appear which are called *indels*. If a multiple of three indels are inserted or deleted the ORF is still intact, only extended or truncated by the referred amount of aa. Every indel different from the multiple of three will disrupt the ORF, and an entirely different protein is transcribed beyond this mutation. Indels are more frequent in genomic non-coding regions than in coding ones (Lemey et al., 2010). A genetic variation can also be caused by recombination, splicing, gene duplication and (in eukaryotes) meiosis.

Calculation and Evaluation of Phylogenetic Trees

A phylogenetic sequence analysis can either be based on nucleic acid sequences or aa sequences, depending on the required resolution of mutations. An aa sequence reflects non-silent mutations only, while a nucleic acid sequence displays the whole collectivity of mutations. The term *substitution rate* represents the number of new mutations over time in a set of compared organisms and plays a crucial role in phylogenetics (von Haeseler and Liebers, 2003). According to the theory of evolution, phylogenetic approaches assume homologous genes in compared sequences (a common ancestor of all organisms). Consequently, closely related genes differ by minor mutations, whereas genes of more distantly related organisms differ by a greater number of substitutions (Britten, 1986; Lemey et al., 2010). The evolutionary relationship between organisms can accordingly be depicted by the degree of difference between their genetic information. This can be visualized as a tree, similar to a pedigree.

To establish a phylogenetic tree, first an alignment is necessary to express differences between the organisms. From the viewpoint of sequence alignment, there are two main methodologies to reconstruct phylogenetic trees from molecular data: They are either based on discrete characters or distance matrices.

Discrete characters consider every position in the alignment as a “character” and every aa or nucleotide as its “state”. Every character is compared independently. Character–state methods can be used to reconstruct ancestral nodes since the original character status of the taxa is retained (Lemey et al., 2010). Distance matrix methods compare the sequences pairwise to produce a distance matrix and a consecutive tree. The usage of an evolutionary model is necessary to correct the simple distance matrix to an evolutionary distance which considers the possibility of more than one mutation at a single point. The advantage of distance methods is the short calculation time when comparing large datasets; its disadvantage is the loss of character–state information – here a reconstruction of ancestral nodes is not possible. The accuracy of the generated trees has to be evaluated in terms of *goodness-of-fit criterions*.

Maximum likelihood (ML) methods consider different possible tree topologies and are based on the statistical probability that the data (and the generated trees) fits the chosen evolutionary model (Tuffley and Steel, 1997). In contrast, *clustering* methods gradually cluster the taxa into a single tree. Most distance matrix methods are evaluated by clustering methods, while character–state methods by the majority employ a goodness-of-fit criterion.

The *neighbor joining* method reconstructs trees aiming to minimize the branch length of all internal branches instead of attempting to cluster the most closely related sequences (Saitou and Nei, 1987). *Maximum parsimony* (MP) reconstructs trees based on the lowest number of steps (mutations) necessary to evolve to the topology based on multiple trees. Though very similar to the ML method, MP trees are not evaluated based on an evolutionary model (Posada, 2008; Tuffley and Steel, 1997). *Bayesian methods* are based on character–state and use goodness-of-fit criteria combined with Markov chain Monte Carlo (MCMC) posterior probability sampling (Huelsenbeck et al., 2001). The tree calculation starts with simulating random parameters of a fictive evolutionary model and calculating the likelihood that these parameters match the data. The next step is the generation of a novel adapted parameter set with consecutive likelihood calculation. The first and second likelihoods are compared and the parameters adjusted accordingly for every forthcoming calculation until hardly any adjustment is necessary. The result is a summary of trees generated, and the first trees have to be discarded as *burn-in* because the parameters were chosen randomly. The answer to the question of which method should be utilized is dependent on the data set, time and expected outcome.

2 STUDY DESIGN

2.1 EXAMINATION OF EUROPEAN BATS FOR THE PRESENCE OF SPECIFIC PATHOGENS

Bats are being increasingly recognized as potential reservoir hosts of emerging and human-pathogenic viruses (1.2.2). While the majority of studies involve viruses harbored by tropical bats, very limited data was available regarding viruses present in European bats. This thesis is contributing to the major goal of a comprehensive study to answer the questions about possible occurrence of zoonotic pathogens in European bats and to characterize them in terms of public health.

2.1.1 Description of the Comprehensive Study

European bats are protected through the European Commission (IUCN, 2013; Red list of endangered species) and through the Agreement on the Conservation of Populations of European Bats (www.eurobats.org). As a consequence, bats cannot be caught and sampled without a special permit. To avoid any further pressure to the threatened bat populations, this study focuses on deceased bats only.

During 2002 and 2009 a total of 486 bats belonging to 19 species were provided by bat researchers and bat carers to the Institute of Zoo and Wildlife Research (IZW) in Berlin for further investigation (Mühldorfer et al., 2011a).

All bats were found dead or moribund and originated from six different regions of Germany (Berlin, Brandenburg, Lower Saxony, Thuringia, Bavaria and Baden-Wuerttemberg). The investigation at the IZW comprised necropsy as well as the histo-pathological and bacteriological investigation (Dr. Kristin Mühldorfer, Dr. Gudrun Wibbelt) (Mühldorfer et al., 2010a, 2010b, 2011a, 2011b). During necropsy eight internal organs were extracted (lung, liver, spleen, salivary gland, intestine, brain, heart and kidney) and transferred to the Robert Koch Institute (RKI, Dr. Andreas Kurth) for all virological examinations, except for the screening for lyssaviruses which was performed at the Friedrich Loeffler Institute (FLI, Dr. Thomas Müller, Dr. Conrad Freuling) (An overview is given in Figure 6).

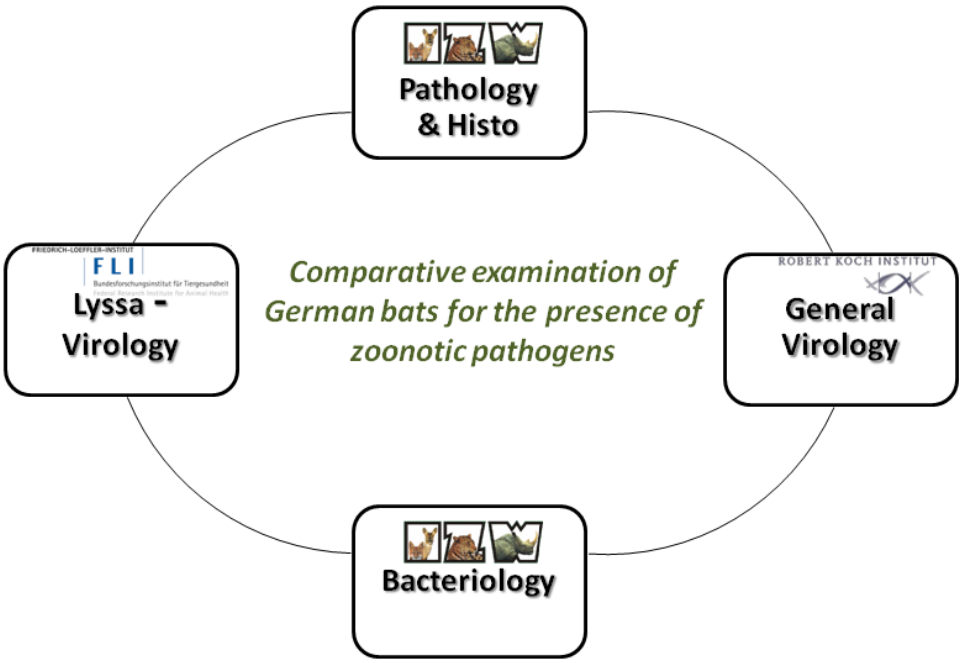

Figure 6 Schematic description of the comprehensive study.

2.1.2 Virological Examination at the RKI

The bat organ samples were examined for viral pathogens by using different approaches, dependent on the sample storage conditions. The internal organs were divided into two aliquots for the virological examinations. One aliquot of the organ was stored in PBS buffer at

-80°C. This aliquot was used for virus isolation by cell culture and DNA virus screening. In the second aliquot stored at -20° C the organ material was supplemented with RNALater® buffer to stabilize viral RNA in the sample. These samples were used for RNA virus screening. An overview of the different virological investigation approaches is given in Figure 7. Cell lines used for virus isolation were chosen regarding their universality (e.g. Vero E6 and Vero B4 cells) and particularity (e.g. R06 cells from *Rousettus aegyptiacus*, PaKi primary cells from *Pteropus alecto*, C/36 insect cells from *Myotis spp*. cell lines) (Crameri et al., 2009; Jordan et al., 2009). Obtained virus isolates were sequenced by high-throughput sequencing to obtain the whole virus genome for further analysis.

The PCR screening for DNA viruses comprised adenoviruses, herpesviruses and poxviruses (Ehlers et al., 1999; Li et al., 2010; Wellehan et al., 2004; Wibbelt et al., 2009). The screening for RNA viruses comprised arenaviruses, bunyaviruses, coronaviruses, filoviruses, flaviviruses, orthomyxoviruses, paramyxoviruses and reoviruses (de Souza Luna et al., 2007; Klempa et al, 2006; Sánchez-Seco et al., 2005; Schulze et al., 2010; Tong et al., 2008; Vieth et al., 2007; Wellehan et al., 2009; Zhai et al., 2007).

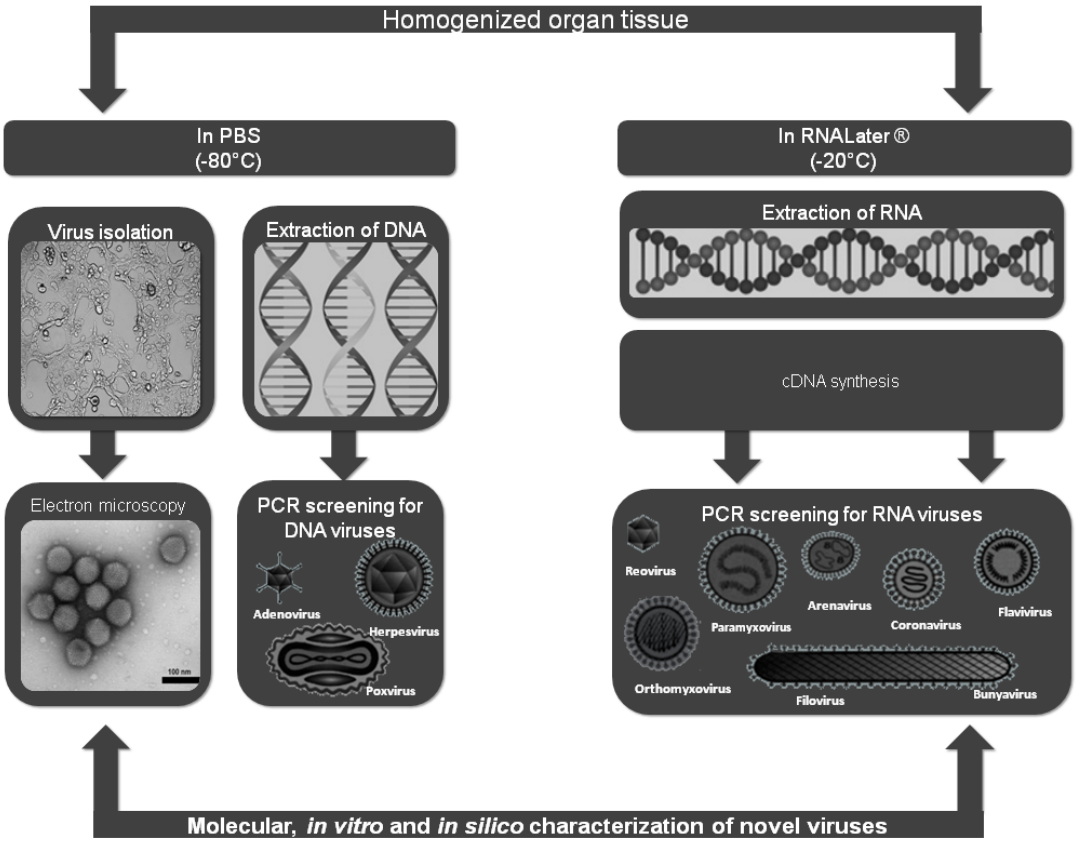

Figure 7 Overview of virological methods performed at the RKI.

2.1.3 Aims of this Thesis within the Comprehensive Study

The thesis presented here was conducted at the RKI under supervision of Dr. Andreas Kurth and PD Dr. Andreas Nitsche as part of the virological examination. The particular aims are illustrated in Figure 8 ([1-6]) and fully described below.

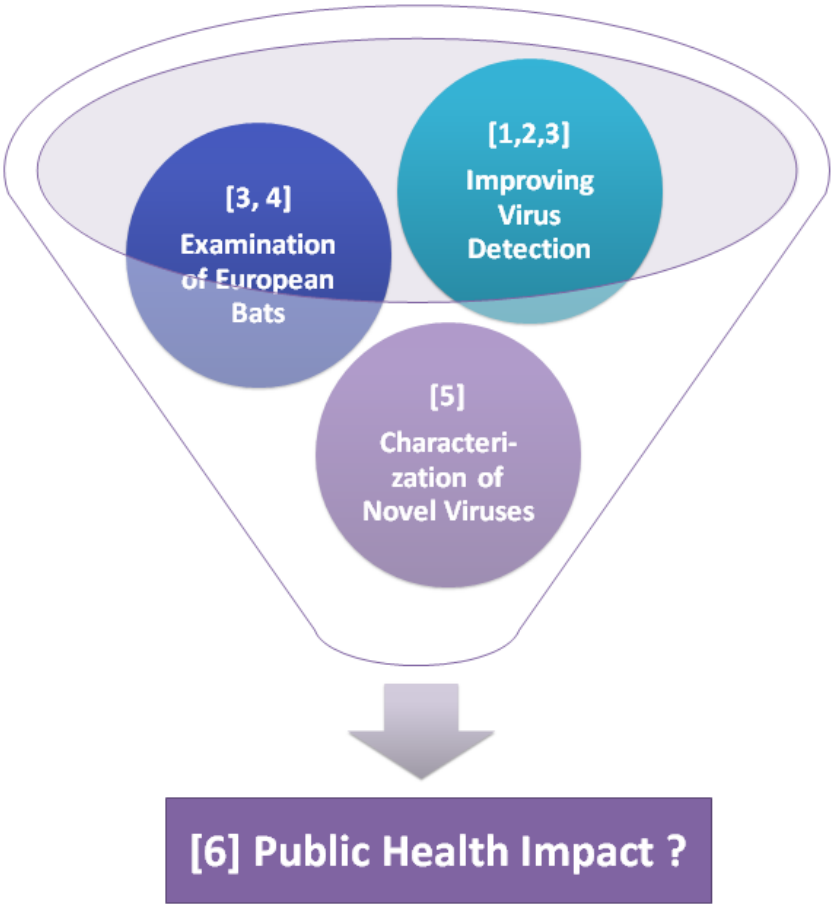

Figure 8 Aims and milestones of the presented thesis. The numbers in brackets refer to the corresponding explanations of the aims.

[1] Improvement of virus detection methods by development of an infected tissue model and a novel purification strategy for viral metagenomic NGS of infected organ tissue (Manuscript V).

[2] Experimental characterization of a novel Australian bat paramyxovirus as visiting scientist at the *Australian Animal Health Laboratory* (AAHL) in Geelong, Australia, to obtain expertise regarding bat paramyxoviruses (Manuscript III) and to exchange virus detection methods (Manuscript in preparation).

[3] Completion of the molecular species determination and of the virological screening for 330 bats and extending the screening for viruses which have not been examined yet (210/330 bats were by then already examined (Mühldorfer et al., 2011a)) including optimization and development of PCR assays and validation methods.

[4] Application of the novel metagenomic NGS method to organ tissue from bats that showed histo-pathological changes, most likely provoked by viral infections, allowing for a consecutive correlation of viruses and pathological genotypes. About 189 organs from 121 bats were selected for viral metagenomic NGS in 9 different pools.

[5] Characterization of novel bat viruses by annotation of the genomes and phylogenetic analysis of isolates obtained and detected (Manuscript I-IV).

[6] Finally, drawing conclusions based on results obtained in this thesis and by other researchers working on European bat viruses, assessing a possible impact on public health (review paper in preparation).

3 SUMMARY OF RESULTS

3.1 PUBLISHED RESULTS OF THIS THESIS

Manuscript I.

Genome analysis of bat adenovirus 2: indications of interspecies transmission. **Kohl, C.,** Vidovszky, M.Z., Mühldorfer, K., Dabrowski, P.W., Radonić, A., Nitsche, A., Wibbelt, G., Kurth, A., and Harrach, B. (2012a). Journal of Virology *86*, 1888–1892.

Manuscript II.

Create two species, Bat adenovirus B and Murine adenovirus B, in the genus Mastadenovirus, family Adenoviridae.

Kohl, C., Vidovszky, M.Z., and Kurth, A. (2011). ICTV 2011.024aV.A.v1.

http://ictvonline.org/proposals/2010.011aV.A.v1.Mastadenovirus-sp.pdf

Manuscript III.

Novel paramyxoviruses in free-ranging European bats.

Kurth, A.*, **Kohl, C.*,** Brinkmann, A., Ebinger, A., Harper, J. A., Wang, L.-F., Mühldorfer, K., and Wibbelt, G. (2012). PloS ONE *7*, e38688.*contributed equally.

Manuscript IV.

Isolation and Characterization of Three Mammalian Orthoreoviruses from European Bats.

Kohl, C., Lesnik, R., Brinkmann, A., Ebinger, A., Radonić, A., Nitsche, A., Mühldorfer, K., Wibbelt, G., and Kurth, A. (2012b). PLoS ONE *7*, e43106.

Manuscript V.

Protocol for Unbiased Virus Detection: Boosting the Signal-to-Noise Ratio for Metagenomics.

Kohl, C., Brinkmann, A., Dabrowski, P.W., Radonić, A., Nitsche, A., and Kurth, A. (2015).

Particular Contribution to the Manuscripts

Table 2 Particular contributions to the manuscripts this cumulative thesis is based on.

Manuscript	Authorship	Concept and establishment of methods	Execution of experiments and analysis	Writing of the manuscript
I.	*First*	80%	80%	80%
II.	*First*	50%	90%	90%
III.	*Shared first*	40%	90%	30%
IV.	*First*	80%	90%	90%
V.	*First*	90%	90%	90%

3.2 MANUSCRIPT I:

http://jvi.asm.org/content/early/2011/11/23/JVI.05974-11.short

3.3 MANUSCRIPT II:

http://ictvonline.org/proposals/2010.011aV.A.v1.Mastadenovirus-sp.pdf

3.4 ABSTRACT MANUSCRIPT III:

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0038688

3.5 MANUSCRIPT IV:

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0043106

3.6 MANUSCRIPT V:

https://www.ncbi.nlm.nih.gov/pubmed/25532973

4 DISCUSSION

The discussion chapter is divided into three sections. First, results of the virus screening are discussed in regard to the applied methods (4.1), followed by the development of novel detection approaches (4.2). Bats as reservoir hosts and the potential risk of zoonotic transmission in Europe are finally discussed in 4.3.

In the comprehensive study (2.1.1) 486 bats were examined for the presence of zoonotic pathogens via pathological, histo-pathological, bacteriological and virological methods.

European bats are on the one hand an endangered species; some are threatened with extinction on the population level and are hence protected (IUCN 2013). On the other hand, bats have been gaining attention increasingly as potential reservoir hosts of some of the most virulent viruses we know so far, while hardly anything is known about viruses in European bats (1.2.2). To overcome the difficulty that any examination might pose a threat to the endangered bat species, the study focused on dead and moribund bats. As a consequence, the study was designed with a pilot character, and hence an analysis regarding virus prevalence is statistically infeasible.

The study could also have been called "*Are **German** bats carriers of zoonotic viruses?*", as all bats originate from different locations in Germany. However, this would not have taken into consideration that European bats are mobile animals, many of them highly abundant and widespread over Europe and migrating throughout the continent (13.1).

This pilot study intended to obtain first results on viruses prevalent in European bat populations. A statistical estimation of prevalence will subsequently become possible: In contrast to this pilot study, the next step would comprise targeted sampling of distinct bat populations throughout Europe. These populations should be examined for a distinct pathogen by preferably non-invasive sampling. Possible selection criteria in doing so are discussed in section 4.1 and 4.3.

4.1 VIRUS SCREENING AT THE RKI

In the presented thesis, the virus screening via cell culture and PCR assays was performed for 330 out of 486 bats belonging to 18 species (Figure 9). The first 156 bats were processed only for bacteriology before the sampling procedure was adapted to allow also for virus detection, resulting in the reduced number of bats also screened for viruses (330/486). Virus detection methods like PCR screening were improved by using the design of experiments (DoE). The priority of the pilot study was to detect viruses that had already been described as zoonotic pathogens and associated with bats (4.1.1, 1.2.2). Hence, the assays for coronaviruses aimed to detect SARS-CoV and MERS-CoV (1.2.2.1), the filovirus assay aimed to reveal Ebola

and Marburg viruses (1.2.2.2), and the paramyxovirus assays aimed to detect henipaviruses (1.2.2.3). The detection of lyssaviruses was performed at the FLI (1.2.2.4) (Mühldorfer et al., 2011b; Schatz et al., 2013).

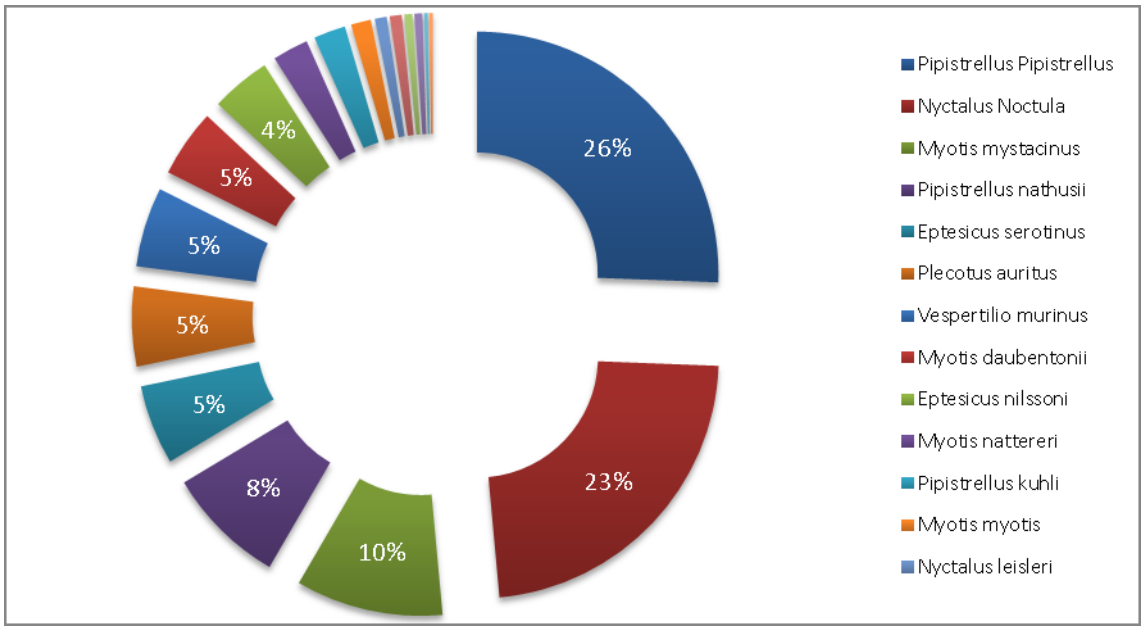

Figure 9 Bat species examined in the presented thesis.

Moreover, broad and sensitive generic virus family-specific PCR assays were chosen to increase the likelihood to find unknown, but related viruses. Additional PCR assays were utilized to detect other highly pathogenic viruses (arenaviruses, hantaviruses, flaviviruses, orthomyxoviruses and poxviruses). A follow-up screening for adenoviruses and orthoreoviruses was initiated after the first successful virus isolation. An overview of PCR assays and respective sample sizes is displayed in Table 3. Virus isolation attempts were performed on different cell lines displayed in Table 4.

4.1.1 PCR Screening for Zoonotic Viruses Strongly Associated with Bats

Bats increasingly have been gaining attention as possible reservoir hosts of highly pathogenic viruses throughout Africa, South-East Asia and Australia, described in 1.2.2 and comprehensively reviewed in (Calisher et al., 2008; Clayton et al., 2012; Dobson, 2005; Halpin et al., 2007; Kuzmin et al., 2011; Newman et al., 2011; Rupprecht, 2009; Rupprecht et al., 2008; Smith and Wang, 2013; Wang, 2009; Wang et al., 2011; Wibbelt et al., 2009, 2010; Wong et al., 2007).

4.1.1.1 Coronaviruses

Viruses of the family *Coronaviridae* can be divided phylogenetically into the alpha-, beta-, gamma- and deltacoronaviruses (Groups 1, 2, 3 and 4, respectively) (Annan et al., 2013; Knipe et al., 2007; Modrow et al., 2010). The alphacoronaviruses comprise the human-pathogenic

coronaviruses (hCoV) hCoV-229E and hCoV-NL63, both causing either apparent or unapparent respiratory infections in humans (Modrow et al., 2010). SARS-CoV (1.2.2.1) and MERS-CoV (1.2.2.1) are members of the betacoronaviruses and, together with the hCoV-OC43 and hCoV-HKU1, are also capable of infecting humans (Modrow et al., 2010).

The PCR assay utilized in this thesis has been described as suitable for all groups of coronaviruses (de Souza Luna et al., 2007). The screening resulted in the detection of one novel alphacoronavirus (Bat CoV 210/09 P.pip) from *Pipistrellus pipistrellus*, with close phylogenetic relationship to viruses described by Gloza-Rausch *et al.* in bats from Northern Germany (Table 3, Appendix Figure 15) (de Souza Luna et al., 2007; Gloza-Rausch et al., 2008). Neither SARS-CoV nor MERS-CoV were detected in the bats examined, nor any other betacoronavirus.

Studies conducted in Bulgaria, Slovenia and Spain described novel alphacoronaviruses and SARS-like-CoV (betacoronaviruses) in bats (Drexler et al., 2010; Falcón et al., 2011; Rihtarič et al., 2010). These SARS-like-CoVs were found predominantly in bats belonging to the family *Rhinolophidae* and additionally within the family *Vespertilionidae* (*Nyctalus leisleri, Nyctalus lasiopterus* and *Miniopterus schreibersii*) (Figure 4, Appendix Table 13.2). However, bats of these families were not examined in the study described here (Figure 9). Because these species tend to be distributed in Mediterranean areas of Europe, no or very limited numbers of the concerned species were investigated in the context of the study described (2.1.1, Figure 9, Appendix Table 13.1). Assuming a host-specificity for these viruses, the likelihood of detecting exactly these SARS-like-CoV in the species examined in this thesis is negligible. However, other SARS-like-CoV might be present in the examined bats.

As previously described (1.2.2.1), bats are not yet proven to be ancestral reservoir hosts of SARS-CoV, and no SARS-CoV have been isolated or detected in bats so far. Nevertheless, SARS-like-CoVs have been detected in European bats. A possible risk for the human population emanating from these viruses remains speculative and is further discussed in section 4.3. Similar to SARS-like-CoV, MERS-like-CoV have been detected in the Netherlands, Germany, Romania (*Pipistrellus spp.*) and Spain (*Hypsugo savii, Eptesicus isabellinus*) (Annan et al., 2013; Cotten et al., 2013; Falcón et al., 2011; Reusken et al., 2013). All novel coronaviruses were detected either from fecal specimens (Annan et al., 2013; Drexler et al., 2010, 2011; Falcón et al., 2011; Gloza-Rausch et al., 2008; Ithete et al., 2013; Reusken et al., 2010; Rihtarič et al., 2010) or oropharyngeal swabs (Falcón et al., 2011), respectively.

Table 3 Results PCR screening

PCR assays	Assay type	Bats #/novel/size*	Assay reference	Novel virus reference
Adenoviridae				
• Adenoviridae (Polymerase)	Pan	79/14/203[H]	(Wellehan et al., 2004)	(Vidovszky et al., 2013)
• Adenoviridae (Hexon)	Pan	15/1/60	Unpublished	Unpublished
• Bt AdV-2	qPCR	12/1/240	(Kohl et al., 2012a)	(Kohl et al., 2012a)
Arenaviridae				
• Old-World	PAN	0/0/60	(Vieth et al., 2007)	-
Bunyaviridae				
• Hanta Virus Puumala	cPCR	0/0/150[a]	In-house design RKI	(Mühldorfer et al., 2011a)
• Hanta Virus Dobrava	cPCR	0/0/150[a]	In-house design RKI	(Mühldorfer et al., 2011a)
• Hanta Virus Tula	cPCR	0/0/150[a]	In-house design RKI	(Mühldorfer et al., 2011a)
• Hantaviruses	Pan	0/0/180[b]	(Klempa et al., 2006)	(Mühldorfer et al., 2011a)
Coronaviridae				
• Coronaviridae	Pan	1/1/240[c]	(De Souza Luna et al., 2007)	unpublished
• Coronaviridae	Pan	0/0/90[a]	In-house design RKI	-
Filoviridae				
• Filoviridae	Pan	0/0/120	(Zhai et al., 2007)	-
Flaviviridae				
• Flaviviridae	Pan	0/0/150[a]	In-house design	(Mühldorfer et al., 2011a)
• Flaviviridae	Pan	0/0/180[b]	(Sánchez-Seco et al., 2005)	(Mühldorfer et al., 2011a)
Herpesviridae				
• BatGHV1	cPCR	1/1/180[a]	(Wibbelt et al., 2007)	(Mühldorfer et al., 2011a)
• BatGHV3	cPCR	7/1/180[a]	(Wibbelt et al., 2007)	(Mühldorfer et al., 2011a)
• BatGHV4	cPCR	22/1/210[a]	(Wibbelt et al., 2007)	(Mühldorfer et al., 2011a)
• BatGHV5	cPCR	11/1/210[a]	(Wibbelt et al., 2007)	(Mühldorfer et al., 2011a)
• BatGHV6	cPCR	24/1/210[a]	(Wibbelt et al., 2007)	(Mühldorfer et al., 2011a)
• BatGHV7	cPCR	2/1/210[a]	(Wibbelt et al., 2007)	(Mühldorfer et al., 2011a)
• BatBHV1	cPCR	1/1/180[a]	(Wibbelt et al., 2007)	(Mühldorfer et al., 2011a)
• Herpesviridae	pan	11/9/60	(Ehlers et al., 1999)	unpublished
Orthomyxoviridae				
• Influenza A	qPCR	0/0/330[d]	(Schulze et al., 2010)	-
Paramyxoviridae				
• Res-Mor-Hen	Pan	2/2/120	(Tong et al., 2008)	(Kurth et al., 2012)
• PAR	Pan	3/3/180	(Tong et al., 2008)	(Kurth et al., 2012)
• BatPV/Myo.mys/E20/09	qPCR	2/1/120	(Kurth et al., 2012)	(Kurth et al., 2012)
• BatPV/Pip.pip/E95/09	qPCR	1/1/120	(Kurth et al., 2012)	(Kurth et al., 2012)
• BatPV/Nyc.noc/E155/09	qPCR	1/1/120	(Kurth et al., 2012)	(Kurth et al., 2012)
Poxviridae				
• Low GC poxviruses	Pan	0/0/263	(Li et al., 2010)	-
Reoviridae				
• T3/Bat/Germany/342/08	qPCR	8/3/120	(Kohl et al., 2012b)	(Kohl et al., 2012b)

pan, Generic family-specific assay; qPCR, quantitative real-time PCR; cPCR, conventional specific PCR; * #/novel/size: #, number of positive samples; novel, number of novel viruses obtained; size, bat sample size; [H]Screening was performed in collaboration with the Hungarian Academy of Science, Balasz Harrach and Marton Vidovsky; [a]All samples belonging to the 210 bats examined prior to this thesis; [b]60 samples belonging to the 210 bats examined prior to this thesis; [c]120 samples belonging to the 210 bats examined prior to this thesis; [d]first 210 samples were examined prior to this thesis.

In the presented thesis, the screening for coronaviruses was performed on internal organ material of the respective bats. The alphacoronavirus detected in this study was obtained from cDNA of pooled organ material (Table 3), and the latter's volume was too limited to allow for independent testing of the corresponding internal organs of the bats.

However, as a proof of principle it has been shown in this study that successful detection of CoV is possible even from organ tissue. To increase the detection likelihood, more generic and sensitive PCR assays can be utilized which are also capable of amplifying MERS-CoV and MERS-like-CoV sequences to reveal the location of replication in the bats' organs. These findings could then be compared with histo-pathological results to clarify whether or not bats are affected by CoV to take a further step towards unveiling the bat reservoir theory.

4.1.1.2 Filoviruses

Ebola virus and Marburg virus are highly virulent and transmissible from human to human and are associated with bats as their reservoir (Pourrut et al., 2009). The discovery of LLOV in French, Spanish and Portuguese Schreiber's bat (*Miniopterus schreibersii*) populations marked the first evidence of natural filovirus occurrence on the European continent (Negredo et al., 2011). Using the assay developed by Zhai *et al.* (Zhai et al., 2007), no filovirus was detected in any of the bats screened in this study. The assay utilized in this study, though powerful in MARV and EBOV detection, could not be evaluated regarding its performance in detecting LLOV, as no target cDNA was available. Further, the primer sequences used in the study by Negredo *et al.* were not provided and thus could not be utilized for the screening. LLOV has been detected in Schreiber's bats which is a Mediterranean species, and due to their spatial abundance was not represented in this study (2.1.1, Figure 9, Appendix Table 13.1).

4.1.1.3 Paramyxoviruses

The natural reservoir hosts of Hendra virus and Nipah virus are fruit bats of the genus *Pteropus*, living in South-East Asia and Australia (Clayton et al., 2012) (1.2.2.3). In this study, two different PCR assays were used to screen for henipaviruses in European bats (Table 3). One assay was developed for the detection of the genera *Respirovirus*, *Morbillivirus* and *Henipavirus* (Res-Mor-Hen), whereas the second one was developed for the detection of all paramyxoviruses (Tong et al., 2008). Neither Hendra virus nor Nipah virus was detected in the bats analyzed in this study.

However, the pan-assays conducted for the *Henipavirus* screening amplified sequences of three formerly unknown paramyxoviruses, two of which clustered into the proposed genus *Jeilong* virus and the third one was related to *Rubulavirus* (Kurth et al., 2012) (Manuscript III).

4.1.1.4 Rhabdoviridae

Two bats (*Eptesicus serotinus*) out of the 486 bats examined in this study were tested positive for EBLV-1. These results were obtained as collaboration between the FLI and IZW and are described here for reasons of completeness (Mühldorfer et al., 2011a; Schatz et al., 2013).

4.1.2 PCR Screening for Other Highly Pathogenic Viruses and Relatives

More than 60 different viruses have been isolated from bats, although only few strongly indicate a zoonotic potential (1.2.2, 4.1.1) (Calisher et al., 2008). Based on publications describing relatives to highly pathogenic viruses in bats, a screening for these pathogens was performed.

4.1.2.1 Arenaviruses

There is only one genus known within the family *Arenaviridae* so far – *Arenavirus* (Modrow et al., 2010). Arenaviruses are divided by their spatial distribution into the Old-World (e.g. Lassa virus, Lymphocytic choriomeningitis virus (LCMV)) and New-World arenaviruses (e.g. Junin virus, Machupo virus, Guanarito virus, Sabiá virus, Flexal virus and Whitewater Arroyo virus) (Modrow et al., 2010). The New-World arenaviruses are further distinguished into line A (North America, South and Central America), lines B and C (both South America) (Charrel et al., 2008). Many of these are capable of causing severe hemorrhagic fevers in humans, and in multiple cases rodents were identified as reservoir hosts (Charrel and de Lamballerie, 2003).

In 1963 the isolation of a novel arenavirus from Jamaican bats (*Artibeus jamaicensis* and *Artibeus lituratus*) was described: Tacaribe virus (Downs et al., 1963). Consecutive serological prevalence studies on Trinidadian and Guatemalan bats of the same spatial area (the Caribbean) confirmed sera of both species to neutralize Tacaribe virus and moreover identified *Sturnira lilium*, *Vampyrops helleri* and the vampire bat *Desmodus rotundus* as possible hosts (Price, 1978; Ubico and McLean, 1995).

The experimental infection of artibeus bats with high doses (10^6 $TCID_{50}$)[4] of Tacaribe virus induced substantive morbidity and mortality in the bats (Cogswell-Hawkinson et al., 2012). Considerably lower doses (10^4 $TCID_{50}$) caused asymptomatic infections and no RNA was detected in the bats' tissues, leading the authors to the assumption that artibeus bats may not be the reservoir host of Tacaribe virus (Cogswell-Hawkinson et al., 2012). However, no human cases of Tacaribe virus infection are described.

[4] $TCID_{50}$ (50% Tissue culture infectious dose)

The screening for Old-World arenaviruses performed in this study did not reveal any hints of the presence of Old-World arenaviruses in European bats. To increase the chance of detecting viruses from the New-World lineage, the utilization of a corresponding PCR assay is desirable. While the screening for arenaviruses in European rodents revealed antibodies against LCMV in northern Italy, Spain and Turkey (Kallio-Kokko et al., 2006; Laakkonen et al., 2006; Lledó et al., 2003), no studies on European bat sera are described. Bat seroprevalence studies for arenaviruses in European bats might contribute to clarify the role of bats in arenavirus infections.

4.1.2.2 Bunyaviruses

The natural reservoir hosts of bunyaviruses are rodents, shrews, voles and arthropods (Ramsden et al., 2009). More than 350 different bunyaviruses have been identified, divided into five differing genera (Modrow et al., 2010):

- *Orthobunyavirus*
- *Phlebovirus*
- *Nairovirus*
- *Hantavirus*
- *Tospovirus*

Hantaviruses are endemic in Europe and cause infections and diseases in humans. Even though rodents, shrews and voles are suspected to be reservoir hosts, the reservoir-question has not been solved conclusively (Heyman et al., 2011; Krüger et al., 2011; Ulrich et al., 2004). The evaluation of the role of European bats in the epidemiology of hantaviruses has been the objective of this screening. The assay utilized in this study was directed to the highly conserved L-segment of hantaviruses. No hantaviruses were detected in any specimen of the examined bats (Table 3).

In humans hantaviruses are causing hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe and the hantavirus cardiopulmonary syndrome (HCPS) in the Americas (Krüger et al., 2011). Andes virus and Sin Nombre virus cause severe HCPS in the Americas, with case–fatality rates up to 35 percent. The strains Seoul and Hantaan circulating in Asia cause HRFS with case–fatality rates ranging from 1 to 10 percent (Krüger et al., 2011). In Europe there are at least five virus strains circulating (Puumala PUUV, Dobrava DOBV, Saaremaa SAAV, Tula TULV and Seoul SEOV) (Heyman et al., 2011). Hantaan virus was found in the lung tissues of *Rhinolophus ferrumequium* and *Eptesicus serotinus* in South Korea. However, in 1995 a further study described that the presence was due to a laboratory contamination (Jung and Kim, 1995; Kim et al., 1994). Hantavirus-related nucleic acids have been detected in a *Nycteris hispida* bat in Sierra Leone, a *Hipposideros pomona* bat in Vietnam, two *Diphylla ecaudata* and *Anoura caudifer* bats in Brazil, two bats (*Neoromicia nanus*) from Côte d'Ivoire and 10 bats (*Pipistrellus*

abramus, *Rhinolophus affinis*, *Rhinolophus sinicus*, and *Rhinolophus monoceros*) from China (Arai et al., 2013; de Araujo et al., 2012; Guo et al., 2013; Sumibcay et al., 2012; Weiss et al., 2012b). Although numerous hantaviruses have been detected in different bat species throughout the world in recent years, one needs to consider that only nucleic acids have been shown. The study by Guo *et al.* assumed an ancestral origin of hantaviruses in the bats, supporting the hypothesis of bats as ancient reservoirs of several virus families. The described studies utilized the same PCR assay used in our study (Klempa et al., 2006). None of the novel hantaviruses were detected in Europe, nor is any of the host species prevalent in Europe. The steep rise of hantavirus infections in Europe (in particular Germany) with more than 2,000 infected humans in a single season, confirms the active replication and maintenance of hantaviruses in the European reservoir host species (Heyman et al., 2011). Hantaviruses are transmissible through urine, feces, dust and other excretion between the reservoir hosts (Ramsden et al., 2009). Bats sharing their habitats with rodents in attics and cellars of buildings are presumably exposed to hantaviruses and could be infected by inter-species transmission. The screening for hantaviruses should thus be extended to bats sampled in peak seasons of hantavirus occurrence in Europe in habitats shared with rodential reservoir hosts.

The majority of orthobunyaviruses (formerly known as Bunyamwera group) are transmitted by gnats, and the genus comprises relevant human-pathogenic viruses like LaCrosse virus and California encephalitis virus, both endemic in North America (Nunes et al., 2005). In Europe orthobunyaviruses have recently gained attention due to the Schmallenberg virus outbreak (Hoffmann et al., 2012). Schmallenberg virus is causing diseases and congenital malformations in livestock, but it is assumed that no zoonotic transmission to humans takes place, based on a serological study conducted on humans who had close contact to infected animals (Ducomble et al., 2012; Hoffmann et al., 2012; Van den Brom et al., 2012). In 1976 a novel orthobunyavirus (Kaeng Khoi virus) was isolated from a bat (*Tadarida plicata*) in Thailand (Williams et al., 1976). A human-pathogenic impact was assumed as miners working in a cave inhabited by bats showed antibodies to Kaeng Khoi virus (Neill, 1985). About 30 years later Kaeng Khoi virus was re-isolated from *Chaerephon plicata* bats in Cambodia (Osborne et al., 2003). Calisher *et al*. reviewed virus isolations from bats worldwide and mentioned the orthobunyaviruses Catu virus, Guama virus and Nepuyo virus isolated from bats (*Molossus obscurus, Artibeus jamaicensis* and *Artibeus lituratus*) (Calisher et al., 2008). Neither are the host bat species endemic in Europe, nor were these isolated orthobunyaviruses reported to cause diseases in humans.

Phleboviruses are transmitted by sandflies and mosquitoes (Phlebotomus group) or ticks (Uukuniemi group) (Giorgi, 1996). Viruses belonging to the genus *Phlebovirus* have been linked

to human diseases; the ones studied best are Toscana virus (TOSV) and Rift Valley fever virus (RVFV). Toscana virus is transmitted by sandflies and ranges among the three most prevalent viruses causing meningitis in the Mediterranean (in particular Italy) during the warm season (Charrel et al., 2005). In 1988 a study described the isolation of Toscana virus from the brain of a *Pipistrellus kuhlii* bat, nevertheless no hemagglutination-inhibiting antibodies were found in the bat's sera and no further detection of Toscana virus in bats has been described ever since (Verani et al., 1988). RVFV is transmitted to humans either vectorial through mosquito bites or by direct contact to infected tissue (Swanepoel and Paweska, 2011). The disease phenotype of RVFV in humans ranges from unapparent to severe courses of hemorrhagic fever and meningoencephalitis (Swanepoel and Paweska, 2011). Although RVFV is endemic in Africa, the outbreaks in Saudi Arabia and Yemen, which were possibly introduced by infected livestock, and the presence of eligible vector species in Europe suggest a potential threat for Europe (Chevalier et al., 2010; Moutailler et al., 2008). RVFV has been isolated from bats of the species *Micropteropus pusillus* and *Hipposideros abae* in the Republic of Guinea (Boiro et al., 1987). The authors further describe the presence of specific antibodies to RVFV in bat sera and assume bats to be the reservoir host of RVFV. In 1999 organ tissues of seven species of bats were examined via serological assays without a positive result (Oelofsen and Van der Ryst, 1999). Within the described study three bats (*Miniopterus schreibersii* and *Eptesicus capensis*) were consecutively infected either orally or intramuscularly with high doses of RVFV (30 ml-100 ml of $10^6 TCID_{50}$). The bats did not show any clinical symptoms, although antigen was detectable within the liver, urine and brown adipose tissues. The authors concluded that bats can be infected by RVFV, and they discuss the reservoir theory (Oelofsen and Van der Ryst, 1999).

It seems reasonable to extend the bat screening to an identification of viruses of the family Bunyaviridae, as bats possibly play a role in the transmission and maintenance of orthobunyaviruses and phleboviruses (except for hantaviruses). A screening for all bunyaviruses may also cover the potential detection of highly pathogenic viruses related to Crimean Congo hemorrhagic fever virus (CCHFV) of the genus *Nairovirus*. CCHFV is prevalent in South-Eastern Europe, Asia, the Middle East and Africa and is transmitted by ixodid ticks (Bente et al., 2013). Cases of CCHFV have increasingly been occurring in Bulgaria, Kosovo, Albania and Greece. CCHFV RNA was also detected in Spanish ticks, underlining the potential of CCHFV to spread also to other countries of the Mediterranean region (Estrada-Peña et al., 2012; Maltezou and Papa, 2010). Ticks are also parasites on bats, hence bats may constitute a possible reservoir for CCHFV introduced into the novel habitats. Pan-bunyavirus PCR assays are available and should be utilized (Lambert and Lanciotti, 2009).

4.1.2.3 Flaviviruses

The genus *Flavivirus* comprises a variety of **ar**thropod **bo**rne human-pathogenic viruses (**Arbo**virus) with a high impact on global health (Modrow et al., 2010). Well-known flavivirus members are Dengue viruses (DENV), Yellow fever virus (YFV), Tick-borne encephalitis virus (TBEV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Hepatitis C virus (HCV) and Hepatitis G virus (GB-V) (Modrow et al., 2010).

The assay used in this study to screen bats did not result in the amplification of a flavivirus (Table 3). Although flaviviruses other than TBEV are distributed mainly in tropical regions, the climate change may shift the abundance of the arthropod vectors and hence eventually the spatial distribution of the virus (McMichael et al., 2006).

Antibodies to DENV were detected in Costa Rican and Mexican bats (*Myotis nigricans, Pteronotus parnellii*, *Natalus stramineus* and *Artibeus jamaicensis* (Aguilar-Setién et al., 2008; Platt et al., 2009). Consecutive infection of *Artibeus intermedius* bats with DENV resulted in histological alteration in bat tissues; nevertheless, virtually no viral RNA was recovered from the internal organs and only 2/24 bats seroconverted, indicating that they were unsuitable hosts for DENV (Perea-Martínez et al., 2013).

In 1970 WNV was isolated from a fruit bat (*Rousettus leschenaultia*) in India (Paul et al., 1970). Subsequent to the epizootic emergence of WNV in the USA, Mexico and Canada, studies on amplification hosts (other than birds) were performed. A seroprevalence study conducted for WNV in the USA revealed low levels of antibodies to WNV in *Eptesicus fuscus* and *Myotis septentriotalis* from Illinois, New Jersey and New York, USA (Bunde et al., 2006; Pilipski et al., 2004). An experimental infection of North American *Eptesicus fuscus* and Mexican *Tadarida brasiliensis* bats resulted in the conclusion that bats were unlikely to serve as amplification hosts of WNV (Davis et al., 2005).

In addition to these cases, a variety of flaviviruses was isolated from bats in Asia and the Americas, overall seroprevalence studies indicated a low prevalence of flaviviruses in the bats' sera and experimental infection showed signs of poor replication (Allen et al., 1970; Cui et al., 2008; Epstein et al., 2010; Kuno and Chang, 2006; Machain-Williams et al., 2013; Main, 1979; Miura and Kitaoka, 1977; Tajima et al., 2005; Watanabe et al., 2010; Yuang et al., 2000).

There are no descriptions of flaviviruses isolated from or prevalent in European bats so far. The poor replication in the host bats' tissues upon experimental infection conflicts with the theory that bats are involved in the sylvatic cycle of arboviral flavivirus transmission (Weaver and Barrett, 2004). However, flaviviruses have been isolated from different bat species in Asia and the Americas, supporting their role as a potential reservoir host (Epstein et al., 2010; Kuno

and Chang, 2006; Miura and Kitaoka, 1977; Paul et al., 1970; Tajima et al., 2005; Yuang et al., 2000).

4.1.2.4 Orthomyxoviruses

The family *Orthomyxoviridae* comprises six genera, three of which are *Influenzaviruses types A, B* and *C* (Modrow et al., 2010). Humans can become infected by all three types of influenzaviruses. While *Influenzavirus type A* can furthermore infect multiple mammalian and avian species, *Influenzavirus type B* is only known to infect seals and *Influenzavirus type C* to infect swine, respectively (Modrow et al., 2010).

Influenzaviruses type A are responsible for global Influenza epidemics every year. Some strains are more virulent than others, resulting in increased morbidity and mortality in humans. For example, the "Spanish Influenza Pandemic" killed more than 50 Mio. people between 1918 and 1920 (Johnson and Mueller, 2002). Influenzaviruses type A are zoonotic, of which the examples of "swine flu" H1N1pan and "avian flu" H5N1 may be the most well-known ones (Garten et al., 2009; Li et al., 2004). Recently, a novel zoonotic influenzavirus type A has been confronting mankind: H7N9, another "avian flu" virus, highly virulent and capable of infecting humans (Gao et al., 2013). So far, all 16 HA types and all 9 NA types building all influenzaviruses type A (making possible 144 combinations of subtypes) have been found in birds – highlighting the avian reservoir as the ancient and natural reservoir of all influenzaviruses type A (Yassine et al., 2010).

However, in 2012 a study reported the detection of influenzaviruses type A from Guatemalan little yellow-shouldered bats (*Sturnira lilium*) (Tong et al., 2012). Interestingly, these novel viruses showed a distinct HA type subsequently referred to as HA17, while the corresponding NA gene was likewise divergent and indicated NA10 (Zhu et al., 2012). Already in 1979 a distinct strain of influenzavirus type A (H3N2) was isolated from common bats (*Nyctalus noctula*) in Kazakhstan (L'vov et al., 1979). These observations led us to investigate influenzaviruses type A in this study. No influenzaviruses were detected with the pan-qPCR assay (Table 3) (Schulze et al., 2010). A novel PCR assay for the detection of influenzaviruses type A was developed with regard to the novel Bat influenzavirus sequences published by Tong *et al*. The sensitivity of the novel assay has yet to be determined.

4.1.2.5 Poxviruses

The family *Poxviridae* is divided into the subfamilies *Entomopoxvirinae* (infecting insects) and *Chordopoxvirinae* (infecting vertebrates) (Van Regenmortel et al., 2000). Several species of poxviruses are capable of infecting humans and domestic animals (Hughes et al., 2010). While humans were the only reservoir for Variola major virus, the natural reservoir hosts of Cowpox virus (CPXV) are assumed to be wild rodents. However, transmission to several accidental

hosts (e.g. humans, cats and elephant) was frequently described (Chantrey et al., 1999; Kurth et al., 2008, 2009; Martina et al., 2006; Ninove et al., 2009; Wolfs et al., 2002). For other strains of poxviruses the zoonotic potential was also demonstrated: Monkeypox virus was transmitted via prairie dogs to humans in the USA (Reed et al., 2004).

Another poxvirus, Vaccinia virus (VACV), known to naturally infect mammalian species, was attenuated for the development of safer vaccines against smallpox (Stickl, 1974). Even though this attenuated virus (modified Vaccinia virus Ankara – MVA) was assumed to be no longer capable of replicating in mammalian cells, it has been demonstrated that cell lines from fruit bats are surprisingly fully permissible for MVA (Jordan et al., 2009). The first poxvirus from a bat was isolated from *Eptesicus fuscus* in the USA in 2011 (Emerson et al., 2013). Shortly afterwards another study described the detection of poxviral nucleic acids from African straw-colored fruit bats (*Eidolon helvum*) through metagenomic sequencing (Baker et al., 2013a). The PCR screening for poxviruses in this study did not reveal any poxviral sequences (Table 3).

4.1.3 Conclusive Statement to PCR Screening

EBLV-1, which was already known to be prevalent in European bat populations, was detected in two serotine bats (Mühldorfer et al., 2011a; Schatz et al., 2013). The screening with generic PCR assays for related viruses (1.2.2, 4.1.1) resulted in the detection of three novel paramyxoviruses, novel adenoviruses and a novel alphacoronavirus (Table 3, Manuscript III) (Kurth et al., 2012). The screening for other highly pathogenic viruses did not result in the amplification of any viral sequence (4.1.2, Table 3).

Like every technique, the application of PCR to detect novel viruses has advantages and disadvantages. In general, the detection of a distinct target via specific primers is sensitive, while the amplification of more diverse sequence regions with degenerate primers is usually less efficient (Compton, 1990). Thus it is necessary to balance the need for a generic amplification using degenerated primers with the need for efficient amplification. The application of nested PCR assays gives relief here, but increases both workload and the risk for contamination. All nested PCR assays basically consist of two amplification rounds, where the second round uses the product from the first one as template to increase the sensitivity (Yang and Rothman, 2004). The usage of different primers in the first and second rounds decreases the risk of smear appearance; hence most assays are composed of three (hemi-nested) or four primers (nested). Accordingly, the primers of the second round have to be designed within the amplification product of the first round to allow amplification. The use of nested PCR requires handling of the first-round PCR product. To give an idea of the difficulties caused by contamination while handling PCR products, a rough calculation is made: Given an initial target

concentration of 10^6 copies/µl, the amplification (30 cycles) in the first PCR round to 10^9 copies/µl is assumed cautiously. The size of a water droplet is physically limited to a diameter of 0.2 mm, and below this it is considered as an aerosol (e.g. fog has an average droplet diameter of 10 µm) (American Meteorological Society, 1959). Setting these assumptions in proportion (i.e. 10^9 copies/ml and 10 µm droplet diameter), an aerosol droplet of PCR product invisible to the naked eye can still contain 500 copies of template, underlining the potential for cross-contamination. The sensitivity of nested PCR assays is often comparable to qPCR assays (~1-10 template copies/µl). Therefore, it is necessary to take precautions (e.g. a separate room to pipette nested PCRs) and to know about the contamination risk when utilizing nested PCR assays.

In the presented study, most of the assays were performed as nested PCR assays to increase the detection likelihood. To avoid false positive results, all positive results were traced back to the initial sample pool and, if positive, consecutively to the individual organs of the examined bats. Furthermore, every amplified product of the right size was sequenced and compared to GenBank for confirmation. Moreover, every assay was carefully evaluated, carrying positive and negative controls.

Hundreds of different PCR assays are available that are potentially suitable to screen for viruses in bats. It was necessary to choose only the most reasonable ones from the broad list of assays due to the limited sample amounts. In the presented study, assays were chosen according to their ability to detect already known viruses of which bats are suspected reservoir hosts (1.2.2, 4.1.1), and also assays were chosen to detect highly pathogenic viruses for which at least a potential role of bats had been described (4.1.2).

Three novel paramyxoviruses, novel adenoviruses and one novel alphacoronavirus were detected, confirming in principle the detection capacity of the described overall approach (Table 3, 4.1.1.1). An extension of the panel of pan-PCR assays might increase the detection likelihood for other viruses: A pan-Bunyavirus PCR seems reasonable considering all the hints pointing to bats (4.1.2.2). For instance, the assays for the detection of the genera *Phlebovirus*, *Nairovirus* and *Orthobunyavirus* published by Lambert and Lanciotti could be utilized (Lambert and Lanciotti, 2009). To improve the screening for flaviviruses, a novel real-time pan-Flavivirus PCR was recently established as a two-step system for the virus screening in bats (Patel et al., 2013). Within this study a novel *Influenzavirus type A* PCR assay was developed in regard to the novel influenzaviruses type A from bats (4.1.2.4) which has to be evaluated before it can be utilized (data not shown). Likewise, a pan-Filovirus assay might be developed for the bat virus screening in regard to the novel LLOV virus, since the pan-Filovirus assay used in this study was developed before the occurrence of LLOV (Negredo et al., 2011; Zhai et al., 2007). In general,

the approach to use already published PCR assays for the screening for novel viruses is a good starting point, but in the case of LLOV and the bat influenzavirus type A the primer sequences were protected by a patent and thus not accessible to the whole scientific community (Negredo et al., 2011; Tong et al., 2012). In addition to the fact that primers that are necessary for screening are not always published, every novel virus sequence changes the requirements for primer specificity for successful detection. Hence it is necessary to stay up-to-date with the primer sequences and adapt them to one's own needs – starting with already published PCR assays, all assays may need to get adapted eventually.

The question why we did not find more viruses in European bats by our PCR screening might be answered in many ways: Either the primers or cycling conditions, even though appropriate for the controls, were not suitable for the yet unknown viruses possibly present in bats. Another explanation could be an insufficient sample quality. However, the storage of the samples in RNALater® and additional testing performed to confirm the integrity of the sample (i.e. via RNA chips on the Agilent Bioanalyzer) contradicts this theory. Admittedly, the RNA integrity was only examined in principle for a limited number of samples, therefore it is not possible to rule out that some of the tissues obtained from the bat carcasses have been degraded to a level where the detection of RNA is no longer possible. Last but not least, these viruses might not be present in European bats and are thus not detectable; this is further discussed in section 4.3: Relevance and Risk Assessment for European Bats.

4.1.4 Virus Isolation

Organ tissues from 330 of 486 bats were inoculated on different cell lines, resulting in four novel virus isolates from European bats, three of which obtained in the context of this study and all four characterized (Manuscript I,II, IV) (Kohl et al., 2012a, 2012b; Sonntag et al., 2009).

4.1.4.1 Cell lines

Successful virus isolation is dependent on the use of susceptible cell lines as well as by the sample quality (1.3.1.1) (Leland and Ginocchio, 2007). When aiming for the detection of novel viruses, it is necessary to choose distinct cell lines from the variety of cell lines available. The *American Type Culture Collection* (ATCC) provides cell lines from more than 150 different animal species, thereof more than 4,000 different lines from humans (www.atcc.org)[5]. It is not feasible to perform cell culture isolation on numerous cell lines simultaneously, as often the sample volume is restricted and the workload increases with every cell line. In this study six different cell lines were chosen for virus isolation attempts (Table 4).

[5]http://www.lgcstandards-atcc.org/en/Products/Collections/Cell_Biology_Collections.aspx (per August 21, 2013)

Table 4 Results virus isolation via different cell culture systems

Cell line	Organism / morphology	#/novel/size*	Reference cell line	Novel virus reference
Permanent (transformed) cell lines				
C6/36	*Aedes albopictus* (larva, whole)	0/0/60	ATCC® CRL-1660™	-
Vero B4	*Cercopithecus aethiops* (kidney)	2/2/60	DSMZ-ACC33	(Kohl et al., 2012b)
Vero E6	*Cercopithecus aethiops* (kidney)	2/2/270	ATCC® CRL-1586™	(Kohl, 2012a; Sonntag et al., 2009)
R05T	*Rousettus aegyptiacus* (fetus head)	0/0/60	(Jordan et al., 2009)	-
R06E	*Rousettus aegyptiacus* (fetus body)	0/0/100	(Jordan et al., 2009)	-
Primary cell lines				
PaKi	*Pteropus Alecto* (kidney)	0/0/120	(Crameri et al., 2009)	-

#/novel/size: #, number of positive samples; novel, number of novel viruses obtained; size, bat sample size

Cell lines from bats (R05T, R06E and PaKi) were utilized to provide a host organism most closely related to the bats examined. Insect cells (C6/36) were chosen because European bats prey on insects and therefore potentially harbor insect viruses. Furthermore, arboviruses replicate well in insect cell lines (Buckley, 1969). Every sample was also inoculated on at least two different cell lines, one of which always was a vervet cell line (Vero B4 or Vero E6). Vervet cells are widely used in virology for virus isolation because of some beneficial attributes: They are susceptible to a broad range of viruses and have often been used in successful virus isolations (Macfarlane and Sommerville, 1969). For instance, SARS-CoV produces the highest titers reported on Vero E6 cells, and this is simultaneously the first known cell line in which a cytopathogenic effect was induced upon infection with the SARS-CoV (Gillim-Ross et al., 2004; Yan et al., 2004). In Vero E6 cells the interferon production is non-functional even though the cells are in general sensitive for interferon; this allows for co-infections with different viruses without interfering effects (Desmyter et al., 1968). The Vero B4 cells show more varying cell morphology compared to Vero E6 cells; apart from this they are very similar.

The sample quality also plays a crucial role and is influenced by appropriate transportation, storage, freezing buffer and temperature applied to the specimens (Leland and Ginocchio, 2007). For instance, the storage of hepatitis C virus (family *Flaviviridae*)-positive specimens at -20°C resulted in a more than 15 percent decrease of detectable RNA within five days, while storage at -80° resulted in a 10 percent decrease within six months (Halfon et al., 1996). For the respiratory syncytial virus (RSV) (family *Paramyxoviridae*) the infectivity was shown to decrease within 28 days at -65°C to merely 10 percent (Hambling, 1964). As a rule of thumb, enveloped viruses feature low tenacities and low temperature stability, while non-enveloped viruses exhibit high tenacity and high stability at different temperatures (Uhlenhaut, 2011). Figure 10 A and B are displaying different virus types with either enveloped or non-enveloped

capsids. In this study viruses were successfully isolated on Vero E6 as well as Vero B4 cells (Kohl et al., 2012b).

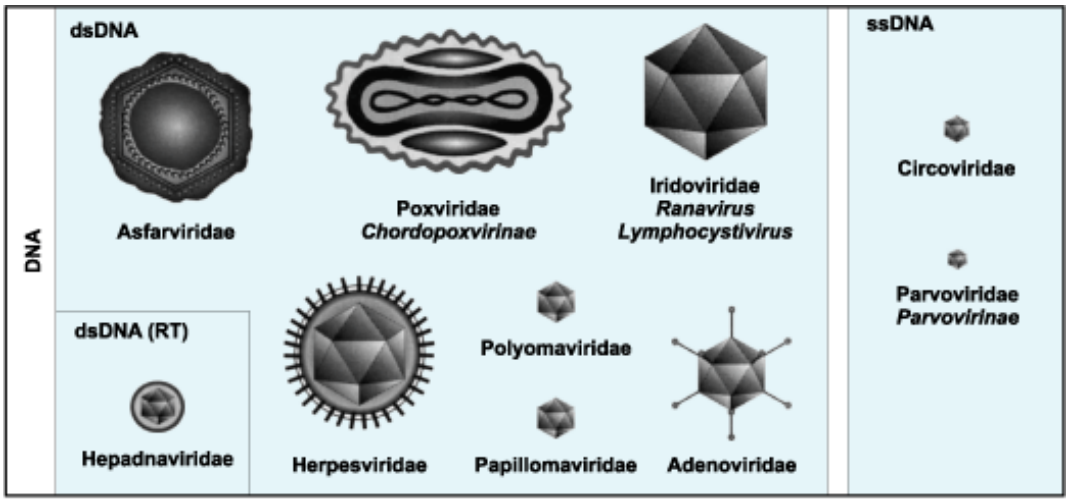

Figure 10 A Families and genera of DNA viruses infecting vertebrates. Individual frames separate taxa of viruses containing double-stranded and single-stranded genomes. Adapted from (Van Regenmortel et al., 2000).

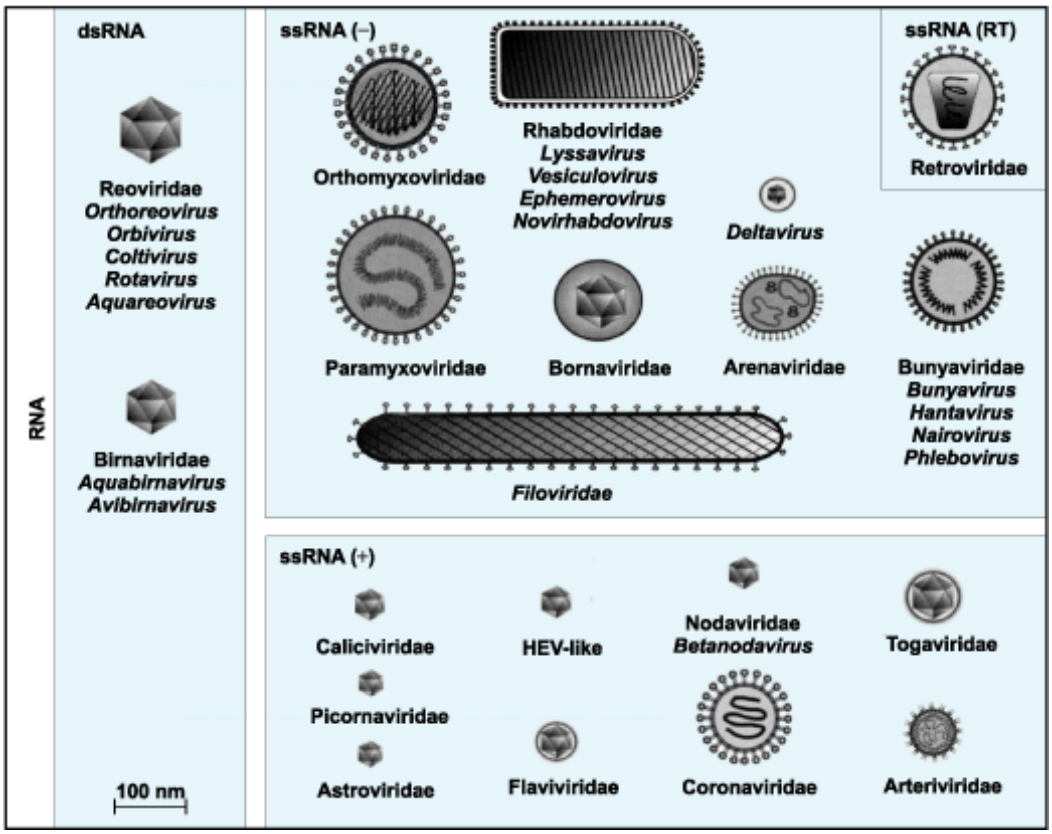

Figure 10 B Families and genera of RNA viruses infecting vertebrates. Individual frames separate taxa of viruses containing double-stranded and single-stranded genomes. Adapted from (Van Regenmortel et al., 2000).

No enveloped viruses have been isolated in this study, which might be due to the low tenacity and temperature tolerance featured by these viruses. The samples were obtained from bat carcasses kept frozen at -20°C until necropsy and extraction of the internal organs, constituting a first freeze-and-thaw cycle (2.1.1). The samples for virus isolation were allotted

to aliquots and stored deep frozen at -80°C. Thawing for virus isolation completed the second freeze–thaw cycle (2.1.2). The initial storage at -20°C as well as the two freeze-and-thaw cycles may have inhibited infectivity of any enveloped viruses potentially present.

4.1.5 Conclusive Statement to Virus Isolation

In addition to the difficulties and restrictions regarding cell culture susceptibility, the necessary freeze-and-thaw cycles might have prohibited the isolation of enveloped viruses (e.g. paramyxoviruses described in Manuscript III (1.3.1.1) (Kurth et al., 2012)). Nonetheless, novel non-enveloped viruses were successfully isolated (Table 4) (Manuscript IV) (Kohl et al., 2012b; Sonntag et al., 2009). Although the stability of non-enveloped viruses is comparably less affected by freeze-and-thaw cycles, successful isolation is not warranted. A consecutively conducted PCR screening for viruses in the bats resulted in identifying additional bats infected with Bat-AdV-2 and orthoreoviruses (Table 3). Possibly, the number of infectious particles was reduced in the positive bats and hence did not result in further virus isolations. Although in theory a single virus particle is enough to detectable infect a host cell, this sensitivity also depends on the sample quality and was outpaced by nucleic acid detection. However, the isolation of viruses is still the gold-standard in virology as it allows in contrast to PCR detection for the investigation of cause-and-effect of a disease (further discussed in section 4.3.1: Henle-Koch-postulates).

Overall, the virus isolation strategy utilized in this study worked well for the isolation of novel viruses from organ tissue. Doubtlessly, the storage and preparation of the specimen should be improved to increase the likelihood of isolating enveloped viruses. Inoculation of all other cells (except for vervet cells) did not result in successful virus isolation. No cell line from bats with defective interferon response is available today. Such cell lines may provide a valuable amendment to vervet cells for the virus isolation from European bats. Viruses that induced a CPE in cell-culture have successfully been detected, but viruses that have not caused a visible effect in cell-culture may have been missed during the screening. NGS is becoming more and more cost-effective and the examination of all inoculated cells by NGS, independent from visible CPE, may solve the problem in the future.

4.2 Viral Metagenomics

A major goal of the presented thesis was the improvement of virus detection by developing and establishing a novel detection strategy (1.3.2.3, 2.1.3). An unbiased virus purification protocol was developed and evaluated for subsequent metagenomic sequencing:

***T**issue-based **u**niversal **vi**rus **d**etection for **v**iral **m**etagenomics* (TUViD-VM) (Manuscript V) (Kohl et al., 2013).

4.2.1 Development of the TUViD-VM Protocol

Classical virus detection methods either require knowledge of the expected pathogen in advance or are limited by the number of feasible approaches and thus presuppose experience, coincidence and a small portion of luck (1.3.1). Classical approaches used in this study resulted in the isolation and the detection of novel bat viruses (4.1); however, it is very difficult to determine whether more viruses might be present in the bat specimens, or whether they are simply not detectable with the approach, though very likely other undetected viruses are present. Electron microscopy with its 'open view' is a very valuable technique for virus identification (1.3.1.1), while sensitivity is too low and the effort necessary is too high for it to serve as a routine diagnostic tool (Laue and Bannert, 2010). NGS can likewise be considered as an 'open view' technique, as every sequence present in the sample will in theory be sequenced unbiased and simultaneously (1.3.2.2). However, data analysis can be extremely time-consuming.

4.2.1.1 Advantages and Limitations of TUViD-VM

The TUViD-VM protocol was developed as part of this thesis and overcomes the limitation of the classical virus detection approaches (1.3.1, 4.1.3, 4.1.5) as it allows for universal virus detection without requiring prior knowledge of suspected viruses from tissue (Manuscript V) (Kohl et al., 2013). As described in 1.3.2.3, the metagenomic detection of viruses in clinical specimens is difficult. Thus the TUViD-VM protocol was developed to overcome these difficulties and was evaluated with poxvirus (dsDNA, enveloped), paramyxovirus (negative-ssRNA, enveloped), reovirus (dsRNA segmented, non-enveloped) and orthomyxovirus (negative-ssRNA, segmented, enveloped), representing a broad range of different virus types (Figure 5, Figure 10 A and B). However, it is conceivable that other virus types may not be detected as efficiently as the ones used to establish the TUViD-VM protocol. A limitation of the TUViD-VM protocol lies in the requirement for undamaged virus particles. During the TUViD-VM purification, the virus particles are separated according to their buoyant density in sucrose by ultracentrifugation. The sample quality is therefore a crucial parameter. Samples should be used fresh or deep-frozen at -80°C or in liquid nitrogen while avoiding any unnecessary freeze-and-thaw cycles. It is not yet determined if stabilizing agents might have a positive effect on the particle stability in tissue samples preceding TUViD-VM purification, but this question is already subject of further studies at the RKI.

4.2.1.2 Controls for TUViD-VM

The TUViD-VM protocol comprises a sequence of individual steps, some of which more fault-prone than others. When a sample is processed with the TUViD-VM protocol it is not possible to check the sufficient and correct purification before applying NGS. The viruses that might be present in the sample are unknown, thus not easily detectable by PCR. The host genome might not be utilized either, as the TUViD-VM protocol decreases the amount of detectable host nucleic acids below the qPCR detection limit. Moreover, a negative NGS result would raise the question of a possibly failed TUViD-VM preparation.

The appliance of a control in terms of an external or internal standard contributes to reliability and validation of positive and especially negative results. To evaluate the functionality of the TUViD-VM preparation, either an internal or an external standard can be used. The advantage of the internal standard is the direct dependency of the standard results with the sample results (until NGS). As an internal standard, intact virus particles could be spiked into the sample, but the disadvantage of spiking with virus particles is the competition of the internal standard nucleic acids with the target nucleic acids in NGS. Hence, the internal standard might decrease the detection likelihood of the viruses present in the examined sample. Apart from these disadvantages, viruses spiked into tissue do not represent a real infection as the spiked viruses are not bonded on cell membranes nor are they encapsulated by cells.

In contrast, an external standard would not compete with viruses in the examined specimens as it is processed separately. It is not necessary to apply NGS to an external standard as its only purpose is to validate the proper TUViD-VM preparation and it can be discarded after testing by qPCR. At best, the external standard consists of authentic infected tissue to mimic the situation in the examined specimen. The disadvantage is already expressed by the word *external*, as it can never be absolutely assured that two samples are treated exactly the same way.

From these options the development of an external standard was chosen as a control for the TUViD-VM protocol. For this purpose embryonated chicken eggs were infected with Sendai virus (SeV, paramyxovirus) and livers were extracted after seven days of incubation. Following determination of the viral copy number by qPCR, the organs were pooled and allotted to aliquots of final volumes of 100 µl containing approx. 100 copies SeV/µl. The aliquots were further tested for consistency by qPCR and found to reveal hardly any variation. The TUViD-VM protocol was applied on four aliquots in two different runs. The results were consistent between all compared runs, which thus further validated the TUViD-VM protocol as a reliable approach. The chosen concentration of 100 copies SeV/µl was proved to be optimal; on the

one hand just above the detection limit, hence very sensitive to possible errors, and on the other hand low enough to minimize the risk of cross-contamination.

4.2.2 Preliminary Results: Bat Viral Metagenomics

The TUViD-VM protocol was developed on model organ tissue of embryonated chicken eggs with the objective to develop a tool for the bat virus screening. After the successful establishment the protocol was applied to bat tissue. Among the 486 bats examined in this study 84 percent (n=408) showed histo-pathological alterations, with the lung as the predominately affected organ (Mühldorfer et al., 2011b). Of these 84 percent, 189 organs from 121 bats showed histo-pathological alterations not associated with bacterial infections or other known diseases and are thus suspected to be of viral etiology (personal communication Dr. Gudrun Wibbelt) (Figure 11) .

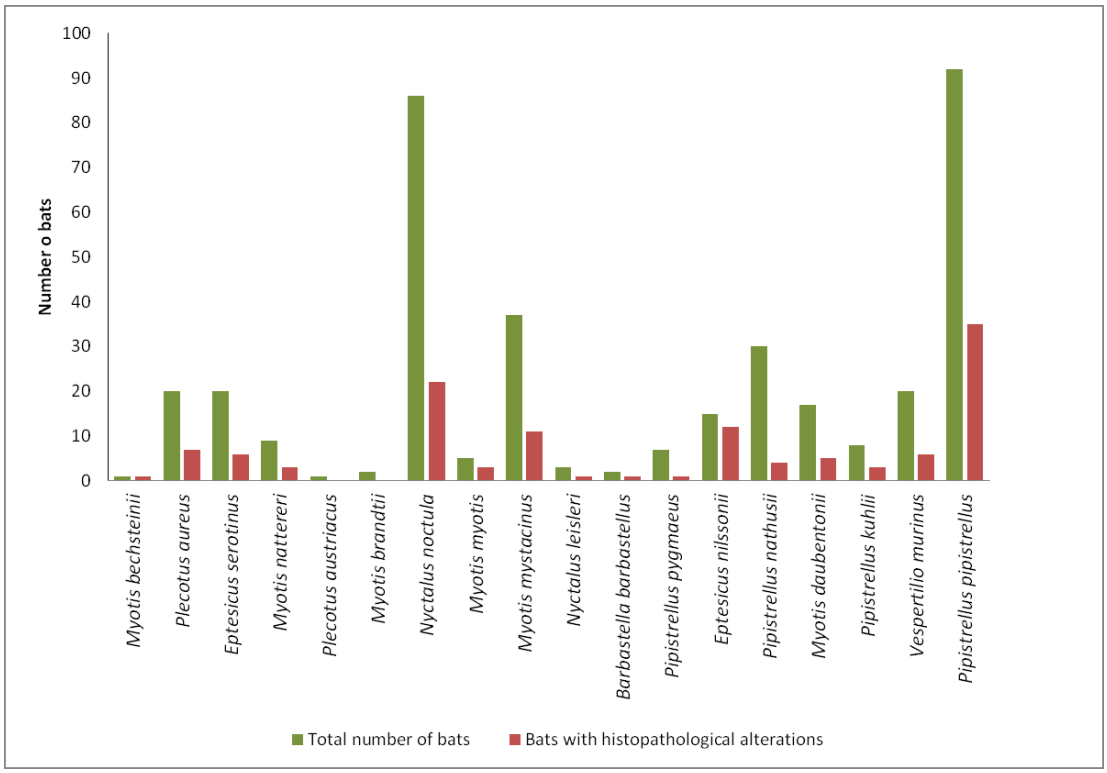

Figure 11 Histo-pathological alterations per bat species examined in this study.

The 189 organs were partitioned into nine different pools for economic reasons. All apportioned pools consisted of approx. 20 organs of either a single bat species or a mix of different species when insufficient numbers of individuals were available.

The TUViD-VM protocol was applied to one of the pools (#8) containing organs from *Plecotus aureus* and *Pipistrellus nathusii*. This pool was chosen for sequencing because it contained intestinal tissues from bat number E342/08 from which the reovirus strain T3/bat/Germany/342/08 was initially isolated during this thesis (Manuscript IV) (Kohl et al., 2012b). The sequencing resulted in more than 80,000 reads (out of 12 mio reads) specific for

strain T3/bat/Germany/342/08 (identity >95%). After identification, the pool and individual organs that pool #8 consisted of were tested back for T3/bat/Germany/342/08. Indeed, positive results were found in the pooled tissue (Ct 34, ~10 copy/ml[6]) and sample E342/08 intestine (Ct 27, ~1000 copies/ml[6]). Surprisingly the same strain was also found in another bat E104/09 (*Pipistrellus nathusii*) that had not been tested for reoviruses before (Ct 36, ~1 copy/ml[6]) (Table 3). The complete metagenomic analysis of the nine bat pools was not included in the presented thesis due to time constraints. These findings highlight the sensitivity and applicability of the TUViD-VM protocol in the detection of novel viruses.

4.3 Relevance and Risk Assessment for European Bats

This section is divided into three chapters; first the general requirements are discussed for linking a pathogen to diseases, followed by the discussion of bats in general as reservoir hosts of emerging viruses and finally the assessment regarding a possible public health risk of viruses obtained in the presented thesis, together with reports from the literature.

4.3.1 Principle of Cause-and-effect: The Henle–Koch Postulates

The postulates drafted by Jacob Henle and Robert Koch in the late 19th century constitute a kind of legislature regarding the principles of cause-and-effect in microbiology (Koch, 1932). Back then it was comparatively straightforward to limit cause-and-effect to four postulates, as neither viruses had been discovered nor was molecular biology developed yet (Table 5).

Table 5 Henle–Koch postulates of cause-and-effect of diseases in microbiology (Koch, 1932).

Original Henle–Koch postulates
1. **The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.**
2. **The microorganism must be isolated from a diseased organism and grown in pure culture.**
3. **The cultured microorganism should cause disease when introduced into a healthy organism.**
4. **The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.**

The second postulate is hard to fulfill for viruses, as they do not grow on nutrient media, but require living cells for replication. In 1937 Rivers proposed the use of cell culture and special pathogen-free animals to extend the postulates for virology (Rivers, 1937). Some approaches were made to draw more modern postulates (Evans, 1976; Falkow, 1988; Fredericks and Relman, 1996). The novel approaches are not widely accepted as recent studies still cite the Henle–Koch postulates (amended by Rivers) as the dictum of cause-and-effect

[6] Homogenized organ tissue: Average size of tissue piece 8mm³ homogenized in 1 ml PBS.

examination also in virology (Breitschwerdt et al., 2013; Fouchier et al., 2003; Osterhaus et al., 2004; Terpstra et al., 1991).

When looking for viruses on a molecular level, it is necessary to consider that only the first postulate is accomplishable. Studies identifying a host–pathogen relationship solely on the molecular level do not take into consideration that detection is not equal to causation. Only a small number of mammalian genomes are yet available. While the release of novel genome sequences during the last decade has been speeding up, also the amount of newly discovered genomic endogenous viral sequences has been increasing (e.g. Belyi et al., 2010; Feschotte and Gilbert, 2012; Holmes, 2011; Horie et al., 2010; Kapoor et al., 2010; Katzourakis and Gifford, 2010). Endogenous virus sequences are integrated into the host genome's junk areas and hard to distinguish from exogenous viruses by simple sequence amplification. Until today, only three bat genomes out of more than 1,100 species of bats worldwide are available (Seim et al., 2013; Zhang et al., 2013). Even though PCR screening and metagenomic studies are indispensable and valuable tools, virologists should stay close to the Henle–Koch postulates when assuming possible virulence of viruses detected in bat hosts.

4.3.2 Bats as Reservoir Hosts

Bats are very unique animals in many ways (1.1.1) and have frequently been suspected as reservoir hosts of an increasing number of viruses (1.2.2, 4.1.2). In this section the theory of bats as reservoir hosts and the relevance of virus detection in bats are discussed.

4.3.2.1 Are Bats a Special Reservoirs of Zoonotic Viruses?

A reservoir host for a disease is defined as a species that is essential for the maintenance and transmission of an infectious agent (Haydon et al., 2002; Olival et al., 2012). Speculations about the role of special features of bats have arisen alongside their increasing recognition as reservoir hosts of highly pathogenic viruses (Calisher et al., 2006, 2008; Dobson, 2005; Omatsu et al., 2007; Wang et al., 2011; Wibbelt et al., 2010) (1.1.1). The flight, allowing for dispersal over long distances, their special immune system (1.1.1.7) and the exceptionally long life-span (1.1.1.3) has been suggested to facilitate viral persistence, as well as torpor and hibernation (1.1.1.5) are discussed as factors in suppressing viral replication (Luis et al., 2013). Furthermore, certain bat species are highly sociable and aggregate to huge colony sizes, also to colonies of mixed bat species, allowing for ready transmission between individuals (Appendix table 13.1) (Calisher et al., 2006; Luis et al., 2013). As bats are some of the most ancient mammals in the context of evolution (1.1.3), it has been assumed that viruses of bats may use cellular receptors that are highly conserved among mammals, and thus these viruses may be highly transmissible to other mammals (Calisher et al., 2006). In comparison to other

mammals, the prolonged co-evolution of bats with their viruses makes them marvelous reservoirs. They seem to be less affected by viral diseases as a result of the co-evolution that allowed for the interrelated adaptation of the bat's immune system alongside the adaptation of the viruses to their bat hosts (1.1.1.7) (Woolhouse et al., 2002).

However, is it true that bats are harboring a higher number of viruses than other animal reservoirs? Or is the increased discovery of bat viruses caused by a sampling bias, encouraged by the initial identification of bats as reservoirs of e.g. Hendra virus? A study based on statistics conducted by Luis *et al.* found that bats host significantly more zoonotic viruses per species than rodents (Luis et al., 2013). Because there are approximately only half as many bat species than rodent species recorded, overall less zoonotic viruses were found in bats than in rodents (Luis et al., 2013). Furthermore, sympatry[7] within the taxonomic order as well as greater body masses and longevity were identified as major traits for the occurrence of zoonotic viruses in bat populations (Luis et al., 2013). Bat species giving birth to more than one individual per year are also increasingly being associated with zoonotic viruses, assuming a connection between sex hormones and immunity (Klein, 2000; Luis et al., 2013). To conclude: The answer is yes! Bats in general are harboring a greater number of zoonotic viruses than the other important reservoir hosts: rodents.

4.3.2.2 Biodiversity, Biogeography and Virus Emergence

More than 1,100 bat species are described worldwide; although they are abundant over the whole world except for the polar regions, a steep diversity gradient is present from the tropics towards the poles (1.1.3) (Buckley et al., 2010; Simmons, 2005b; Teeling et al., 2005; Willig and Selcer, 1989b). Are fewer viruses prevalent in European bats because of the lower abundance of species in the more temperate Europe? And is the zoonotic risk posed by bats accordingly decreased?

Only few studies on the biogeography of microorganisms are available. These studies found that the latitudinal diversity gradient has either no or a top–down effect on microbial diversity (Buckley et al., 2003; Fierer and Jackson, 2006; Hillebrand and Azovsky, 2001; Martiny et al., 2006; Peterson, 2008). Two studies hypothesized that the local diversity and dispersal of viruses is very high, though overall the viral diversity is limited on the global scale (Breitbart and Rohwer, 2005; Dolan, 2006). Therefore, no assumptions regarding viral diversity in species abundant in temperate climate can be taken. As the total number of abundant species is not essential, the change in biodiversity may play a role.

[7] Sympatry is the overlapping of habitats between populations of different species of the same distinct taxa.

The effect of decline in biodiversity on the emergence of diseases is subject of numerous publications (Keesing et al., 2010; Mills, 2006; Ostfeld, 2009; Randolph and Dobson, 2012; Salkeld et al., 2013; Woolhouse and Gowtage-Sequeria, 2005; Woolhouse et al., 2001). There are basically arguments for two theories; reduced biodiversity could either increase or decrease the risk of disease transmission (Keesing et al., 2010): On the one hand, the availability of hosts for pathogens is crucial and is influenced by host abundance, behavior and host condition. When host species are intermixed with other species, as a consequence the infection risk and the pathogen load is reduced due to competition, also known as 'dilution effect' (Keesing et al., 2010; Mitchell et al., 2002; Salkeld et al., 2013). On the other hand, the more species serve as hosts, the more is there contact potential for disease transmission (Keesing et al., 2010; Saul, 2003). Vice-versa, the majority of medically important pathogens are capable of opportunistically infecting multiple hosts (Woolhouse and Gowtage-Sequeria, 2005; Woolhouse et al., 2001).

Mills discussed the effect of biodiversity on the basis of hemorrhagic fever viruses in rodents and proposed that anthropogenically disturbed habitats (low-diversity habitats) present the greatest risk: First of all, disturbed habitats are often inhabited by opportunistic species which serve as hosts of hemorrhagic fever viruses, and secondly because of the decreased 'dilution effect' in disturbed habitats (Mills, 2006). In contrast, Salkeld *et al.* describe in their meta-analysis (conducted on all kinds of pathogen emergence where biodiversity was reported) that the ecology of infectious diseases is often too complicated to draw general assumptions and found only weak evidence supporting the 'dilution effect' theory (Salkeld et al., 2013). On the other hand, based on the examples of West Nile virus and Lyme disease, Ostfeld *et al.* showed that indeed high biodiversity strongly reduces the transmission risk (Ostfeld, 2009).

However, for pathogens already prevalent in host populations, the decrease of biodiversity frequently resulted in higher transmission rates (Keesing et al., 2010; Morse, 1995). For almost half of the zoonotic diseases newly emerged by spill-over since 1940, a preceding change in land-use, agriculture and wildlife hunting was reported (Keesing et al., 2010) (Figure 12). All of the above-mentioned effects contribute to changes in biodiversity and increased contact between human and animal hosts, also in Europe. For instance, in certain African and Asian countries bats are traded, hunted and eaten as bush meat (Figure 12), which might result in an increased transmission risk of viruses prevalent in bats (Mickleburgh et al., 2009; Wolfe, 2005). Once spill-over in novel hosts has occurred, a high density of the novel host population facilitates eventually the establishment in the novel niche. Thus, the human overpopulation together with decreased biodiversity might be mutual factors giving rise to the establishment

of emerging infectious diseases. To conclude, the Baas–Becking hypothesis might still be appropriate: Everything is everywhere, but the environment selects (Becking, 1934).

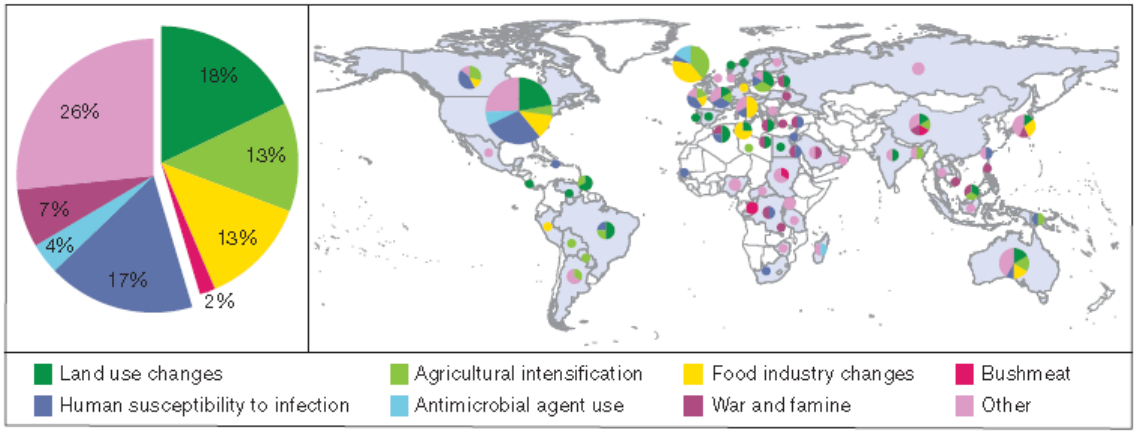

Figure 12 Adapted from (Keesing et al., 2010): Drivers and locations of emergence events for zoonotic infectious diseases in humans from 1940–2005. Left panel, worldwide percentage of emergence events caused by each driver; right panel, countries in which the emergence events took place, and the drivers of emergence.

4.3.3 Zoonotic Bat Viruses in Europe?

Viruses of many different families have been detected in European bats (Appendix Table 13.2). Some of the families comprise viruses that are considered highly pathogenic for humans and zoonotically transmissible, e.g. viruses of the families *Filoviridae*, *Paramyxoviridae*, *Rhabdoviridae* and *Coronaviridae*. Hence, all virus families were exemplarily detected containing the key virus examples where bats are suspected to be reservoir hosts (1.2.2). Beyond controversy, these viruses are some of the most virulent strains we know so far (EBOV, MARV, Hendra virus, NiV, lyssaviruses, MERS-CoV). Some of these viruses were found in both Megachiroptera and Microchiroptera (EBOV, lyssaviruses); others are restricted to either Mega- (HeV, NiV) or Microchiroptera (MERS-CoV). Nevertheless, antibodies to HeV and NiV have been detected in various species of bats (Clayton et al., 2012; Li et al., 2008; Peel et al., 2013). Even though Megachiroptera are rarely abundant in Europe, the presence of their viruses in Microchiropteran species cannot be ruled out.

Although frequent studies report the presence of SARS-like-CoV and MERS-like-CoV sequences in European bats (Annan et al., 2013; Drexler et al., 2010; Falcón et al., 2011; Ithete et al., 2013; Rihtarič et al., 2010), no final conclusion can be drawn regarding their zoonotic potential. A related virus is not necessarily considerable as zoonotic or pathogenic. To stay with the SARS-CoV example, it has been described that few alterations in the SARS-CoV spike protein compared to related bat CoV enabled binding to its host receptor ACE-2 and thus became capable of infecting humans (Li et al., 2006). So far, the SARS-like-CoV viruses detected in bats all lack these alterations and thus are not predictable in their capacity of infecting humans. Although strains are similar or related on a nucleic acid level, the distinct function of

proteins is crucial when determining the host range: Therefore, merely similarity is not enough to examine the potential of viruses to infect humans or even predict their virulence. Most importantly, no SARS-like-CoV or MERS-like-CoV was ever isolated from bats, nor was a single transmission of SARS-like-CoV or MERS-like-CoV to humans reported, and there is no hint yet that these viruses of bats pose a threat to public health. The case of MERS-CoV is slightly different, as a sequence with 100 percent similarity was detected in a bat (*Taphozous perforates*[8]) in Saudi Arabia (Memish et al., 2013). This finding initiated a controversy among leading CoV experts, as the journal *Nature* recently reported (Mole, 2013). First the complete genome sequence of MERS-CoV should be obtained from the bat to confirm that it is indeed MERS-CoV and not coincidentally just a short conserved region of 190 nt. Furthermore, a prevalence study might bring insights into the distribution of MERS-CoV in bat populations. Although *Taphozous perforates* is not abundant in geographical Europe, climate change and environmental factors may have an effect on future distribution of this bat species (Figure 13) (Van der Putten et al., 2010). The case of MERS-CoV emergence is arrestingly demonstrating the necessity of virus discovery and prevalence studies. With the first sequence of MERS-CoV that became available, bats were suspected as reservoir hosts, not only because MERS-CoV is a SARS-CoV relative. They were also suspected because previous bat virus discovery studies provided eligible sequences of bat CoV to GenBank allowing for correlation with the novel MERS-CoV. As already discussed for SARS-like-CoV, the mere sequence information obtained from one host species cannot predict the pathogenicity and virulence in another host species.

Hence, the demonstration alone of sequences of a novel filovirus and novel paramyxoviruses does not provide evidence of a possible public health threat (Drexler et al., 2012a; Kurth et al., 2012; Negredo et al., 2011). The novel bat paramyxoviruses are not related to viruses of the highly -pathogenic genus *Henipavirus* (Manuscript IV III) (Drexler et al., 2012a; Kurth et al., 2012). There is no evidence suggesting whether or not the novel paramyxoviruses and filovirus are capable of infecting humans. Following the Henle–Koch postulates, they should be isolated and further characterized to learn about the evolution of paramyxoviruses and filoviruses with their bat hosts. But as filoviruses are described as highly pathogenic for humans and the novel LLOV virus belongs phylogenetically to the Ebola-like genus, the occurrence of this virus should be carefully observed in prevalence studies in the highly abundant *Miniopterus schreibersii* (Figure 14).

[8] The species identification performed was not free of doubt as it was based on exclusion criteria (no Cytochrome b sequence of *Taphozous perforates* is available in GenBank (Memish et al., 2013)).

Figure 13 Geographical distribution of *Taphozous perforates*. (IUCN 2013)

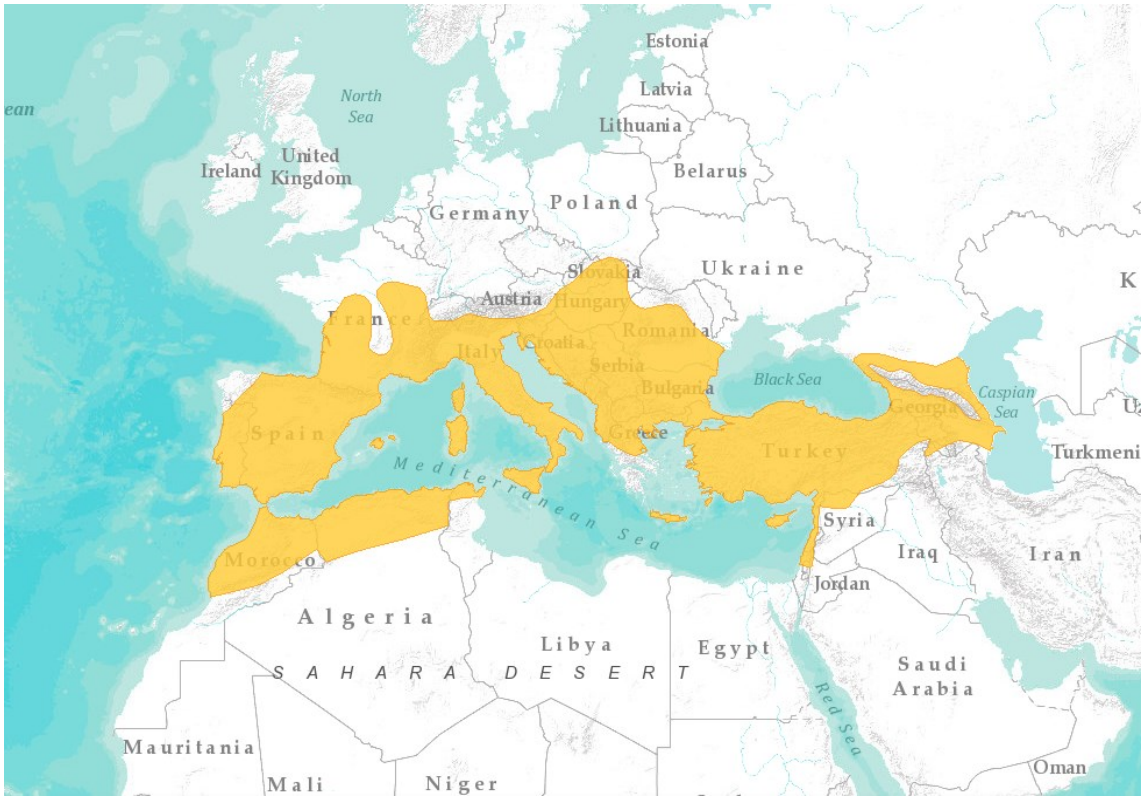

Figure 14 Geographical distribution of *Miniopterus schreibersii* in Europe. (IUCN 2013)

So far, the only virus isolates obtained from European bats allowing for broader investigation are TOSV and orthoreoviruses, both from Italy, together with the four virus isolates obtained in this study and of course the four previously isolated lyssaviruses (discussed

further down) (Kohl et al., 2012b; Lelli et al., 2013; Schatz et al., 2013; Sonntag et al., 2009; Verani et al., 1988).

However, the TOSV was isolated from a bat's brain in 1988, while simultaneously isolating TOSV from sandflies in the lab (Verani et al., 1988). As TOSV has never been reported from bats afterwards and no hemagglutination-inhibiting antibodies were initially found in the bat's brain, there is a reasonable chance that this TOSV isolation may have been a cross-contamination. However, bats are also affected by ecto-parasites and hence could in principle get infected by TOSV accidentally (Zahn and Rupp, 2004).

Bat AdV-2 was isolated in this study and the whole genome was obtained and circumstantially discussed in Manuscript I and Manuscript II (Table 4) (Kohl et al., 2011, 2012a). In summary, strong evidence is provided suggesting an ancestral inter-species transmission of Mastadenoviruses between bats and canids. The closely related canine AdV contribute to the severe kennel cough syndrome in canids (Buonavoglia and Martella, 2007).

The whole genome of one of the three orthoreovirus isolates (T3/Bat/Germany/342/08) was likewise analysed and discussed in Manuscript IV (Table 4) (Kohl et al., 2012b). Summing up the data for the reovirus isolates from Germany and Italy, a close relationship to the genus *Mammalian Orthoreovirus* (MRV) is revealed and in particular to an orthoreovirus obtained from a dog (strain T3/D04) with hemorrhagic enteritis in Italy (Decaro et al., 2005; Kohl et al., 2012b; Lelli et al., 2013). No ancestral relationship was assumed here, but rather an opportunistic 'behavior' of the novel MRVs as they were detected in various different bat species in this study. Moreover, the newly isolated MRVs are phylogenetically related to viruses capable of inducing severe meningitis in humans (Tyler et al., 2004). Recently, a study published by Steyer *et al.* described the detection of a T3/Bat/Germany/342/08-like virus from a child hospitalized with acute gastroenteritis in Slovenia (Steyer et al., 2013). The causing agent was determined to be an MRV with highest similarity to T3/Bat/Germany/342/08 isolated in the presented thesis (98.4–99.0 percent in the respective segments) (Steyer et al., 2013). This might indicate human-pathogenic potential for strain T3/Bat/Germany/342/08, thus the case of SARS-CoV showed that even little changes are important to determine the host range, this has to be determined in further studies. Interestingly, no contact was reported between the infected child and bats, but contact to a domestic dog was assumed (Steyer et al., 2013). Like in the case of Rabies virus, which was prevalent in both bats and terrestrial mammals (e.g. dogs, raccoons, skunks and foxes) of the Americas, a continuing exchange and transmission between bats and canids or other terrestrial animals might be possible (Davis et al., 2006). The isolated viruses allow for a seroprevalence study (cross-reactivity and cross-neutralization with other strains) in humans, which shall be initiated to examine the

prevalence of specific antibodies to Bat MRVs in Germany and Italy (where these viruses have been found) to clarify their zoonotic potential. This is especially interesting as also Asian bat orthoreoviruses of the genus *Pteropine Orthoreovirus* have already been linked to potentially zoonotic respiratory diseases in humans (Chua et al., 2007, 2008).

Rhabdoviruses of the genus *Lyssavirus* that have been found in Europe are considerably harmful and truly zoonotic agents, causing inevitably the death of unvaccinated humans that are not treated in time before onset of the rabies disease (Johnson et al., 2010) (1.2.3.10, 13.1). Even though bat-transmitted rhabdoviruses have a lethality of virtually 100 percent and are suspected as transmissible by bat biting and scratching, the total European human fatality number reported is low (n=2–5 since 1963) (Johnson et al., 2010; Racey et al., 2013). All described hosts of EBLV-1 and EBLV-2 are synanthropic, hence sharing their habitats with humans (Racey et al., 2013). EBLV-1 has been predominantly detected in *Eptesicus serotinus* and *E. isabellinus* in Europe, both living in buildings, roofs and attics predominantly in the southern regions of Europe (*E. serotinus* until 55°North, *E. isabellinus* in southern Portugal[9]), and male bats are reported to co-roost with multiple bat species (Appendix table 13.1) (Dietz et al., 2007). EBLV-1 was also reported from *V. murinus, M. schreibersii, M. myotis, M. nattererii, R. ferrum-equinum* and *T. teniotis.* Whether these bat species constitute accidental hosts infected by spill-over from co-roosting *E. serotinus* species, or whether they are additional reservoirs, has not yet been determined (Bourhy et al., 1992; Fooks et al., 2003; Schatz et al., 2013; Serra-Cobo et al., 2002; Van Der Poel et al., 2005).

Two human cases described by Johnson *et al.* were confirmed as infections with EBLV-2 which is prevalent in European *M. daubentonii* and *M. dasycneme* (Johnson et al., 2010; Schatz et al., 2013). *M. daubentonii* is prevalent in North-East Europe and is frequently found co-roosting with *P. pipistrellus* and *M. nattereri*; *Myotis dasycneme* is found throughout Europe and in the Mediterranean, co-roosting with *M. capaccini*. So far none of the co-roosting bats were reported to carry EBLV-2 (Appendix table 13.1) (Dietz et al., 2007); however, spill-over transmission to other animals (stone-marten, sheep and cat) was described for EBLV-1.

This might constitute an eventually enzootic maintenance of EBLV-1 in other species and thereby increase the risk of zoonotic transmission (Dacheux et al., 2009; Müller et al., 2004; Schatz et al., 2013; Tjørnehøj et al., 2006). An experimental infection of red foxes with EBLV-1 resulted in neurological symptoms in all of the infected foxes, although no EBLV-1 RNA was detectable in the brain tissues (Vos et al., 2004). A natural spill-over transmission between bats

[9] *E. isabellinus* is a north African population of *E. serotinus* that is controversy but not concluding discussed as a novel species (IUCN and 2013).

and red foxes is conceivable, but whether the natural reservoir range of EBLV-1 could be extended to red foxes is questionable[10]. Two novel bat lyssaviruses detected (BBLV and WCBV) are hard to evaluate regarding their human-pathogenic potential, as no human cases have been reported so far (Freuling et al., 2011; Kuzmin et al., 2005).

Overall, lyssaviruses prevalent in European bats pose a risk to public health, and preventive measures have already been taken by many European countries (e.g. surveillance, vaccination plans and post exposure prophylaxis) (Racey et al., 2013). But it is necessary to balance the risk with the total number of fatal human cases during the last 35 years (5 cases of 590 million people living in greater Europe) (Racey et al., 2013). Accordingly, the risk is relatively low and would probably fall to zero if people were educated appropriately: Contact with certain bat species might be risky and requires post exposure prophylaxis afterwards. Only few of the European bat species are known to be reservoirs of EBLV-1 and EBLV-2, but all of the European species are endangered or close to extinction. Relocation or culling of bat colonies, although an obvious solution from the viewpoint of the public, increases the risk of lyssavirus exposure and transmission and shall not be considered (Streicker et al., 2012). Only education can channel public fear to avoid further threats to the bats and the public, respectively.

[10] Red foxes have been the natural reservoir hosts of the Rabies virus in Europe, before it was eradicated in European populations of carnivores (Anderson et al., 1981).

5 CONCLUSIONS AND FUTURE PROSPECTS

5.1 CONCLUSIONS

The objective of the presented thesis was the examination of European bats for zoonotic pathogens to allow for a first assessment of European bats as reservoirs of zoonotic viruses. The examination was part of a comprehensive pilot study comprising virology, bacteriology, pathology and histo-pathology.

The presence of viruses in European bats was demonstrated in the presented thesis. The classical detection methods (PCR and cell-culture screening) were improved, resulting in an increased virus detection likelihood. Moreover novel detection approaches were developed and are now available for unbiased detection of known and novel viruses by high-throughput-techniques (NGS). This can not only be utilized for bat tissues, but for all tissue samples obtained through the investigation of outbreaks of unknown etiologies in human and animals.

Until now, lyssaviruses have been the only proven zoonotic viruses in European bats and may cause the Rabies disease in humans upon bites or scratches from bats. However, only few bat species are known to transmit lyssaviruses in Europe, and the number of human cases is considerably low. Nevertheless, education of the public shall be intensified to avoid any unnecessary and easily preventable infection. Although viruses with zoonotic potential have been detected in European bats, no clear assumption can be drawn without further studies. Sero-prevalence studies should be conducted on the reoviruses isolated in the context of this study, especially as closely related viruses were detected in a diseased child in Slovenia. Other bat viruses should be isolated (e.g. MERS-like-CoV) to allow for follow-up sero-prevalence studies.

In general, bats are special reservoir hosts because of their biological features, long-time co-evolution and high diversity of viruses that can be found. Furthermore, there is neither a clearly decreased risk in the emergence of zoonotic viruses in temperate climate compared to the tropics nor a decreased risk in regions of lower biodiversity.

In conclusion, drivers of emergence in the tropics have also validity in Europe. On the other hand, European bats are endangered species and some are threatened by extinction. Although lyssaviruses are prevalent in European bats, and some viruses might have potential, the overall hazard for humans is comparably low. However, the protection of bats (and any wildlife) will consecutively protect the general public.

5.2 FUTURE PROSPECTS

- The TUViD-VM protocol will be applied to cases of infectious diseases with unknown etiology to unveil diseases with an 'open view'. Similarly, a purification strategy for electron microscopy could be developed in the future, based on the TUViD-VM protocol and subsequently be named TUViD-EM. Histo-pathological and virological results could be correlated and further confirmed by immuno-histology.
- The TUViD-VM protocol could be further refined and improved to allow also for the simultaneous detection of bacterial pathogens.
- The TUViD-VM protocol was applied to organ tissues of European bats that displayed alterations conspicuous for viral infections. Viruses in these tissues that have not been detected with the methods applied in the presented thesis might be discovered in this way.
- PCR assays should be continuously improved to react and adapt to the increasing sequence information available.
- A seroprevalence study in humans could be conducted in collaboration with Slovenian and Italian researchers to elucidate the zoonotic potential of the orthoreoviruses that have been isolated from bats in Germany and Italy and a child in Slovenia.
- The bat screening should be extended to include species in the Mediterranean regions and islands in Europe. The approach should comprise non-invasive sampling (urine, feces, oral swabs and ecto-parasites) and sera sampling.
- Sequences obtained during this thesis that showed insufficient similarity to any sequence in GenBank will be re-checked with the growing database on later occasions. Further developments in the field of bioinformatics may also allow a better assessment of such reads in the future, permitting a reanalysis and detection of hitherto unrecognized viruses in the data.

6 ABSTRACT

Bats have been increasingly recognized as reservoir hosts of highly pathogenic and zoonotic emerging viruses (Marburg virus, Nipah virus and Hendra virus). While in the beginning of the study numerous studies focused on tropical regions, little was known on pathogens of European bats. The presented thesis was conducted as part of the virological examinations within a comprehensive study examining European bats for zoonotic pathogens by virology, bacteriology, pathology and histo-pathology.

Virus detection approaches comprised PCR screening for numerous highly pathogenic virus families by generic assays and virus isolation with different cell lines. The screening resulted in the detection of novel adeno-, corona- and paramyxoviruses and novel orthoreovirus isolates. The full genome sequence of one orthoreovirus and a previously isolated adenovirus was obtained, annotated and phylogenetically analyzed. To improve virus detection from tissue, a novel virus purification protocol (TUViD-VM) was developed and extensively validated with metagenomic *Next Generation Sequencing*. The results confirmed the dramatically increased unbiased detection likelihood for both known and novel viruses. Consecutively, the TUViD-VM protocol was applied to bat tissues displaying histo-pathological alterations related to viral infections and will be bioinformatically analyzed in future studies.

To conclude, no evidence for the presence of zoonotic viruses in addition to the already known lyssaviruses has been found in any of the bats. Although novel viruses were detected, their zoonotic potential remains unclear unless further studies are conducted. At present, it is assumed that the risk posed by bats to the general public is considerably low. Anyhow, most European bats are at least endangered, and the increasing public fear of bats and their viruses is an obstacle to their protection. Educating the public regarding bat lyssaviruses might result in reduced threats to both the public and the bats.

7 ZUSAMMENFASSUNG

Fledermäuse wurden in den letzten Jahren vermehrt als Reservoir hochpathogener zoonotischer Erreger identifiziert (Marburg-Virus, Nipah-Virus, Hendra-Virus). Während sich zu Beginn der Arbeit die meisten Studien auf die tropische Regionen konzentrierten, war über Viren in europäischen Fledermäusen nur wenig bekannt. Die vorliegende Arbeit wurde als Teil der virologischen Untersuchungen zur Erfassung der zoonotischen Erreger in europäischen Fledermäusen mittels Virologie, Bakteriologie, Pathologie und Histopathologie erstellt.

Der Nachweis von Viren erfolgte durch die Anwendung von generischen Screening-PCRs, passend auf verschiedene Familien hochpathogener Viren sowie durch Virusisolation mit unterschiedlichen Zelllinien. Es wurden bisher unbekannte Adeno-, Corona- und Paramyxoviren mittels PCR nachgewiesen, sowie bisher unbekannte Orthoreoviren isoliert. Die komplette Genomsequenz eines isolierten Orthoreovirus und eines vor Beginn der vorliegenden Arbeit isolierten Adenovirus wurden generiert, annotiert und phylogenetisch analysiert. Um die Nachweismethoden ausgehend von infiziertem Gewebe zu verbessern, wurde eine neue Virusaufreinigungsmethode (TUViD-VM) entwickelt und durch Metagenomsequenzierung umfassend validiert. Die Ergebnisse der Validierung bestätigten die deutliche Steigerung der Nachweiswahrscheinlichkeit für bekannte und neuartige Viren. Nachfolgend wurde das TUViD-VM-Protokoll an histopathologisch auffälligem Fledermausgewebe mit Verdacht auf Virusinfektion angewendet, und in späteren Studien wird die bioinformatische Analyse erfolgen.

Zusammenfassend wurde kein Beweis für das Vorhandensein zoonotischer Viren (außer Lyssaviren) in den untersuchten Fledermäusen gefunden. Obwohl bisher unbekannte Viren detektiert wurden, muss deren zoonotisches Potential erst durch weitere Studien geklärt werden. Nach bisherigem Kenntnisstand ist das Risiko, das von Fledermäusen für die Bevölkerung ausgeht, als verhältnismäßig gering einzustufen. Die meisten europäischen Fledermausarten sind in ihrem Bestand zumindest gefährdet, so dass die wachsende Angst der Bevölkerung vor Fledermausviren dem Schutz der Tiere entgegensteht. Die Aufklärung der Bevölkerung bezüglich Fledermaus-Lyssaviren könnte die Gefahr für die Bevölkerung und die Fledermäuse gleichermaßen verringern.

8 TABLES AND FIGURES

8.1 TABLES

TABLE 1 OVERVIEW ON CURRENT NGS TECHNOLOGIES 22
TABLE 2 PARTICULAR CONTRIBUTIONS TO THE MANUSCRIPTS THIS CUMULATIVE THESIS IS BASED ON 34
TABLE 3 RESULTS PCR SCREENING 42
TABLE 4 RESULTS VIRUS ISOLATION VIA DIFFERENT CELL CULTURE SYSTEMS 53
TABLE 5 HENLE–KOCH POSTULATES OF CAUSE-AND-EFFECT OF DISEASES IN MICROBIOLOGY 59

8.2 FIGURES

FIGURE 1 TEMPORAL PATTERN OF BAT DIVERSIFICATION 5
FIGURE 2 SPECIES RICHNESS MAP FOR BATS AND OTHER SPECIES THAN BATS 6
FIGURE 3 NUMBER OF BAT SPECIES PER 500 KM X 500 KM, CORRELATED TO THE LATITUDE 7
FIGURE 4 BAT SPECIES CORRELATED TO VIRAL SPECIES DETECTED IN EUROPEAN BATS 15
FIGURE 5 OVERVIEW ON GENOME CHARACTERISTICS AND ORIENTATION OF MAIN VIRUS FAMILIES 21
FIGURE 6 SCHEMATIC DESCRIPTION OF THE COMPREHENSIVE STUDY 29
FIGURE 7 OVERVIEW OF VIROLOGICAL METHODS PERFORMED AT THE RKI 30
FIGURE 8 AIMS AND MILESTONES OF THE PRESENTED THESIS 31
FIGURE 9 BAT SPECIES EXAMINED IN THE PRESENTED THESIS 40
FIGURE 10 A AND B: FAMILIES AND GENERA OF VIRUSES INFECTING VERTEBRATES 54
FIGURE 11 HISTO-PATHOLOGICAL ALTERATIONS PER BAT SPECIES 58
FIGURE 12 DRIVERS AND LOCATIONS OF EMERGENCE EVENTS FOR ZOONOTIC INFECTIOUS DISEASES 63
FIGURE 13 GEOGRAPHICAL DISTRIBUTION OF *TAPHOZOUS PERFORATES* 65
FIGURE 14 GEOGRAPHICAL DISTRIBUTION OF *MINIOPTERUS SCHREIBERSII* IN EUROPE 65
FIGURE 15 PHYLOGENY OF THE NOVEL ALPHACORONAVIRUS DETECTED IN THIS STUDY 95

9 LITERATURE

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10 LIST OF PUBLICATIONS

11 CONTRIBUTIONS TO CONFERENCES

12 CURRICULUM VITAE

13 Appendix

13.1 TABLE A1: CHARACTERISTICS OF EUROPEAN BAT SPECIES

Family	Species	Distribution	Weight / age	Roosting preferences	Roost interference	Characteristics
Pteropodidae	Egyptian rousette (*Rousettus aegyptiacus*)	Cyprus, Tenerife, Southern Turkey	1,500 g 25 years	Caves, 50-500 individuals, nursery roots	All cave-dwelling bats	No hibernation, feed on fruit seasonal migration?
Rhinolophidae	Lesser horseshoe bat (*Rhinolophus hipposiderus*)	Central and Southern Europe	7 g 21 years	Forest, close to water broad spectrum of habitats: buildings, roofs, bridges and funnels, 10-200 individuals	*Myotis myotis*, *Myotis emarginatus*, *Plecotus* spp.	Average age 4-5 years, territorial (<20 km)
	Greater Horseshoe bat (*Rhinolophus ferrumequinum*)	Central and Southern Europe	24 g 30.5 years	Agriculture areas, Mediterranean ranges, Northern elongation: roofs Southern elongation: caves, funnels 20-1,000 individuals	*Myotis emarginatus*, *Rhinolophus* spp., *Miniopterus schreibersii*, *Myotis capaccinii*	Cluster, feeds on beetles, slowly flying, territorial (<100 km)
	Mediterranean horseshoe bat (*Rhinolophus euryale*)	Southern Europe	14 g 13 years	Limestone caves, northern elongations: roofs, bunker, cellars 20-1,000 individuals	*Miniopterus schreibersii*, *Myotis capaccinii*, *Myotis emarginatus*, *Myotis myotis*	Winter cluster up to 2,000 individuals, isolated colonies of more than 10,000 bats territorial (<50 km)
	Mehely's horseshoe bat (*Rhinolophus mehelyi*)	Very south of Europe	17 g 12 years	Limestone caves, mines 20-500 individuals, winter roosts up to 4,500 bats	Often *Rhinolophus euryale*, other cave-dwelling bats	Territorial (< 90 km)
	Blasius' horseshoe bat (*Rhinolophus blasii*)	South-Eastern Europe	14 g unknown	Limestone caves, mines, underground, 30-3,000 individuals	*Myotis myotis*, *Myotis blythii*, *Rhinolophus euryale*, *Rhinolophus mehelyi*, *Rhinolophus ferrumequinum*, *Miniopterus schreibersii*, *Myotis capaccinii*	Territorial (<10 km)
Vespertilionidae	Daubenton's bat (*Myotis daubentonii*)	Whole of Europe until 63°North	10 g 30 years	Forests, parks, orchards, hollow tree trunks, bat boxes, vault cracks, expansion joints of bridges; winter: bunker, caves, cellars, 20-600 individuals	*Myotis capaccinii*	Changing tree trunks every 2nd to 5th day. Hibernation with up to 20,000 individuals, Migrating between winter and summer roosts (<150 km), up to 300 km

Family	Species	Distribution	Weight / age	Roosting preferences	Roost interference	Characteristics
						reported
	Pond bat (*Myotis dasycneme*)	North-Eastern Europe	18g 20.5 years	Roofs of churches, roof veneering, possibly hollow tree trunks, bat boxes, winter: caves, bunker, cellar 20-300 individuals	*Myotis nattereri*, *Pipistrellus pipistrellus*	Migrating <350 km between summer and winter roosts
	Long-fingered bat (*Myotis capaccinio*)	South Eastern Mediterranean regions	10 g unknown	Year-round limestone caves, close to water, individuals in rock cracks, bridges, buildings, 30-500 individuals	*Miniopterus schreibersii* and other cave-dwelling bats	Winter roosts: up to 50,000 individuals Migrating (<100 km)
	Brand's bat (*Myotis brandtii*)	Central and Eastern Europe	7g 28.5 years	Bat boxes, hollow tree trunks, wooden facades, roofs, close to forests; winter roosts in caves and mines, 20-60 individuals (up to 200 bats)	*Pipistrellus pygmaeus*, *Pipistrellus nathusii*	Territorial (<40 km)
	Whiskered Bat (*Myotis mystacinus*)	Whole of Europe until 64°North	7 g 23 years	Cracks in buildings, window blinds, facades, behind tree bark; winter: caves and mines, 20-60 individual bats	*Pipistrellus pipistrellus*, single individuals from other species	Average age 3.5-5 years Change their roosts every 10th to 14th day. Territorial (50-100 km)
	Steppe whiskered bat* (*Myotis aurascens*)	Bulgaria (20 km² area)	9 g unknown	Expansion joints of bridges	Unknown	unknown
	Alcathoe whiskered bat* (*Myotis alcathoe*)	Spots of Europe, mainly France	5.5 g unknown	Tree cracks, behind oak bark, very close to water (<100 m); winter: caves	*Myotis* spp., *Plecotus* spp.	unknown
	Natterer's bat (*Myotis nattereri*)	Whole of Europe until 60°North	10g 21.5 years	Bat boxes and hollow tree trunks, in buildings, more southernly in stone cracks, bridges; winter: cracks, caves, cellars	Unknown	Territorial (<40 km)
	Geoffroys's bat *Myotis emarginatus*)	Southern and Western Europe	9 g 18 years	Roofs, churches, buildings, cattle sheds; winter: underground	*Rhinolophus* spp., *Myotis myotis*, *Myotis capaccinii*, *Miniopterus schreibersii*	Territorial (<40 km)
	Bechstein's bat (*Myotis bechsteinii*)	Central and Eastern Europe	10 g 21 years	Hollow tree trunks, cracked trees, bird and bat boxes; winter: underground or in tree trunks, 10-50 individuals	Mostly solitaire, sometimes with *Myotis nathereri*, *Myotis daubentonii*	Very territorial
	Greater mouse-eared bat (*Myotis myotis*)	Europe, except for the UK and Scandinavia	27 g 25 years	Roofs and attics, cellars; winter: caves, mines, single individuals in stone cracks	*Rhinolophus* spp., *Myotis oxygnatus*, *Myotis capaccinii*, *Miniopterus schreibersii*, *Myotis ermaginatus*	Average age 2.7-4.9 years migrating up to 400 km
	Lesser mouse-eared bat (*Myotis oxygnatus*)	Southern Europe	26 g 33 years	Roofs and attics, caves, bunkers; winter: caves or underground	*Rhinolophus* spp., *Myotis capaccinii*, *Miniopterus schreibersii*, *Myotis ermaginatus*, *Myotis myotis*	Average age 14-16 years mixed species clusters territorial (<15 km)

Family	Species	Distribution	Weight / age	Roosting preferences	Roost interference	Characteristics
	Maghrebian mouse-eared bat (*Myotis punicus*)	Islands in the Mediterranean (Sardinia, Corsica, Malta)	25 g unknown	Caves, 1,000 individuals	*Rhinolophus* spp., *Myotis capaccinii*, *Miniopterus schreibersii*	Unknown, assumed territorial
	Noctule bat (*Nyctalus noctula*)	Whole of Europe, except for the very northern and southern areas	30 g 12 years	Woodpecker caves, high hollow tree trunks, in particular beech trees, bat boxes, 20-60 individuals	Unknown	Average age 2.2 years migrate in autumn southwards and back in spring, also in daylight (<1500 km)
	Greater noctule bat (*Nyctalus lasiopterus*)	Spot-like areas in Europe until 50° North	53 g unknown	Hollow tree tunks, bat boxes, attics, up to 80 individuals	*Nyctalus noctula*, *Nyctalus leisleri*, *Pipistrellus pipistrellus*, *Pipistrellus nathusii*	Migrating over large distances, prey on birds
	Leisler's bat (*Nyctalus leisleri*)	Europe until 57° North	18 g 11 years	Woodpecker caves, high hollow tree trunks, bat boxes; winter: roofs, attics, 20-50 individuals	*Nyctalus noctula*, *Nyctalus lasiopterus*, *Pipistrellus pipistrellus*, *Pipistrellus nathusii*, *Myotis bechsteinii*	Seasonal migrating (<1,000 km)
	Azorean bat (*Nyctalus azoreum*)	Azores islands	13 g Unknown	Cracks in buildings	Unknown	Hunts in daylight
	Common pipistrelle (*Pipistrellus pipistrellus*)	Europe until 56° North	7 g 16 years	Buildings, cracks, facades, attics; winter: big colonies in cellars, tunnels and caves, 50-100 individuals, winter: up to 60,000 individuals	Unknown	Average age 2.2 years territorial (<20 km)
	Soprano pipistrelle (*Pipistrellus pygmaeus*)	Europe until 63° North	7 g 8 years	Buildings, cracks, facades, attics, hollow tree trunks, bat boxes; winter: bat boxes, tree trunks, up to 800 individuals	Unknown	Average age 1.2-1.6 years, unknown but migratory behavior assumed
	Nathusius' pipistrelle (*Pipistrellus nathusii*)	Europe until 60° North	10 g 14 years	Behind tree bark, hollow tree trunks, bat and bird boxes, wooden roofs, attics, churches; winter: stacks of wood, tree trunks, up to 20 individuals	*Pipistrellus pipistrellus*, *Myotis brandtii*, *Myotis dasycneme*	Average age 2.5-3.0 years, seasonal migration (<2,000 km)
	Kuhl's pipistrelle (*Pipistrellus kuhlii*)	South-western Europe	8 g 8 years	Stone cracks, building cracks; winter: expansion joints, up to 20 individuals	*Pipistrellus pipistrellus*, *Hypsugo savii*	Territorial
	Madeira pipistrelle (*Pipistrellus maderensis*)	Madeira	7 g unknown	Cracks in buildings, bat and bird boxes, up to 100 individuals, active year-round	Unknown	Territorial active year-round
	Savi's pipistrelle (*Hypsugo savii*) (aka *Pipistrellus savii*)	Southern Europe	9 g unknown	Stone cracks, cracks in walls or buildings, up to 15 individuals	Unknown	Unknown

Family	Species	Distribution	Weight / age	Roosting preferences	Roost interference	Characteristics
	Parti-coloured bat (*Vespertilio murinus*)	Central and Eastern Europe	15 g 12 years	Stone cracks, cracks in walls or buildings, up to 60 individuals	Unknown	2 pups per year
	Serontine bat (*Eptesicus serotinus*)	Europe until 55° North	25 g 24 years	Buildings, cracks, roofs, attics, facades, 10-60 individuals	Male bats share roosts with multiple bat species	Territorial (<50 km)
	Northern bat (*Eptesicus nilssonii*)	Central, Northern and Eastern Europe	13 g 21 years	Sub-roofs, facades, sometimes tree caves, 20-50 individuals	*Eptesicus serotinus*, *Myotis mystacinus*, *Pipistrellus pipistrellus*	Territorial
	Isabelline Serotine Bat (*Eptesicus isabellinus*)	Southern Portugal	16 g unknown	Stone cracks, buildings	Unknown	Assumed migration between Africa and Portugal
	Anatolian serotine bat (*Eptesicus anatolicus*)	Southern Turkey	21 g unknown	Antic ruins, stone wall cracks, bridges, up to 11 individuals	Unknown	Unknown
	Hoary bat (*Lasiurus cinereus*)	Iceland, Orkney islands	38 g unknown	Trees	Unknown	Migrating from Greenland and Northern USA with strong west-winds to Iceland and the Orkney islands
	Barbastelle bat (*Barbastella barbastellus*)	Europe until 58-60° North	10 g 22 years	Forests, behind tree bark, bat boxes, buildings; winter: behind bark, mines, tunnels, ruins, up to 1,000 individuals	Unknown	Average age 5.5-10 years Territorial (<40 km)
	Brown long-eared bat (*Plecotus aureus*)	Europe until 62-64° North	9 g 30 years	Tree and building roosts, bat boxes; winter: underground, tree trunks, caves, 5-50 individuals	*Plecotus austriacus*, *Eptesicus serotinus*, *Rhinolophus hipposiderus*, *Myotis myotis*, *Myotis nattereri*	Average age 4 years territorial (<30 km)
	Alpine long-eared bat (*Plecotus macrobullaris*)	Not well known, alpine regions, Mediterranean islands	10 g unknown	Roofs and attics of churches	Unknown	Unknown
	Sardian long-eared bat (*Plecotus sardus*)	Sardinia	8 g unknown	Limestone caves and attics, up to 12 individuals	Unknown	Unknown
	Grey long-eared bat (*Plecotus austiacus*)	Europe until 53° North	10 g 25 years	Buildings, roofs, attics, Mediterranean: stone walls cracks, expansion joints of bridges, bat and bird boxes; winter: caves, cellars, 10-30 individuals	Unknown	Average age 5-9 years territorial (<62 km)
	Balkan long-eared bat (*Plecotus kolom batovici*)	Eastern coastline of Mediterranean	9 g Unknown	Stone wall cracks, bridges, caves, churches, 15-20 individuals	*Plecotus macrobullaris*	Unknown
	Tenerife long-eared bat (*Plecotus teneriffae*)	Canary islands (Tenerife, La Palma, El	Unknown Unknown	Volcano caves, stone cracks, buildings, up to 37 individuals	Unknown	Unknown

Family	Species	Distribution	Weight / age	Roosting preferences	Roost interference	Characteristics
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Miniopteridae	Schreibers' bat (*Miniopterus schreibersii*)	Mediterranean Europe including islands	14 g 16 years	Limestone caves (year-round), mines, cellars, underground openings; winter: expansion joints of bridges, attics, up to 70,000 individuals	All resident cave-dwelling bat species	Seasonal migration (<900 km)
Molossidae	European free-tailed bat (*Tadarida teniotis*)	Mediterranean Europe including islands	30 g 13 years	Cracked stone walls, ranges, canyons, expansion joints of bridges and buildings, 5-400 individuals	Unknown	Hibernation in Switzerland only (up to 8 days), using visual navigation at dusk and dawn instead of echolocation

13.2 TABLE A2: VIRUSES FOUND IN EUROPEAN BATS

Virus family	Genus	Bat species	Origin	Detection	Reference
Adenoviridae	Mastadenovirus	*Pipistrellus pipistrellus*, *Pipistrellus nathusii*	Germany	Isolation PCR	(Sonntag et al., 2009) (Kohl et al., 2012a)
	Mastadenovirus	*Nyctalus noctula* *Rhinolofum ferrum-equinum*	Hungary	PCR	(Jánoska et al., 2010)
	Mastadenovirus	*Myotis myotis*	Germany	PCR	(Drexler et al., 2011)
Astro-viridae	Mamastrovirus	*Myotis myotis*	Germany	PCR	(Drexler et al., 2011)
Bunya-viridae	Phlebovirus Toscana virus	*Pipistrellus kuhli*	Italy	Isolation	(Verani et al., 1988)
Coronaviridae	Betacoronavirus	*Rhinolophus euryale* *Rhinolophus blasii* *Rhinolophus ferrumequium* *Rhinolophus mehelyi* *Rhinolophus hipposideros* *Miniopterus schreibersii* *Nyctalus leisleri*	Bulgaria	PCR	(Drexler et al., 2010)
	Betacoronavirus	*Rhinolophus hipposideros*	Slovenia	PCR	(Rihtaric etal., 2010)
	Betacoronavirus	*Pipistrellus pipistrellus* *Pipistrellus nathusii* *Pipistrellus pygmaeus*	Germany Ukraine Romania	PCR	(Annan et al., 2013)
	Betacoronavirus Alphacoronavirus	*Pipistrellus pipistrellus* *Myotis daubentonii* *Myotis dasycneme* *Pipistrellus pipistrellus* *Nyctalus noctula*	Netherlands	PCR	(Reusken et al., 2010)
	Alphacoronavirus	*Myotis dasycneme* *M. daubentonii* *Pipistrellus nathusii* *P. pygmaeus*	Germany	PCR	(Gloza-Rausch et al., 2008)
	Alphacoronavirus	*Myotis myotis*	Germany	PCR	(Drexler et al., 2011)
	Alphacoronavirus Betacoronavirus	*Eptesicus isabellinus* *Hypsugo savii* *Myotis daubentonii* *Miniopterus schreibersii* *Myotis myotis* *Nyctalus lasiopterus* *Pipistrellus kuhlii* *Pipistrellus* sp.	Spain	PCR	(Falcón et al., 2011)
Filoviridae	Ebola-like virus	*Miniopterus schreibersii*	Spain	PCR	(Negredo et al., 2011)
Hepe-viruses	Hep-E related viruses	*Eptesicus serotinus* *Myotis bechsteinii* *Myotis daubentonii*	Germany Bulgaria	PCR	(Drexler et al., 2012)
Herpesviridae	Betaherpesvirus Gammaherpesvirus	*Myotis myotis* *Myotis nattereri* *Nyctalus noctula* *Pipistrellus pipistrellus* *Plecotus auritus*	Germany	PCR	(Wibbelt et al., 2007)

Virus family	Genus	Bat species	Origin	Detection	Reference
	Betaherpesvirus Alphaherpes	*Rousettus aegyptiacus*	Hungary	PCR	(Jánoska et al., 2010)
	Gammaherpesvirus	*Eptesicus serotinus*	Hungary	PCR	(Molnár et al., 2008)
Paramyxoviridae	Unassigned	*Myotis mystacinus* *Pipistrellus pipistrellus* *Nyctalus noctula*	Germany	PCR	(Kurth et al., 2012)
	Morbilli	*Myotis bechsteinii* *Myotis daubentonii* *Myotis myotis* *Myotis alcathoe* *Myotis capaccini*	Germany Bulgaria Romania	PCR	(Drexler et al., 2012)
Reoviridae	Orthoreovirus	*Plecotus aureus* *Myotis mystacinus* *Pipistrellus pipistrellus* *Pipistrellus nathusii* *Pipistrellus kuhlii* *Nyctalus noctula*	Germany	Isolation PCR	(Kohl et al., 2012)
	Orthoreovirus	*Pipistrellus kuhlii* *Rhinolophus hipposideros* *Vespertilio murinus* *Tadarida teniotis*	Italy	Isolation PCR	(Lelli et al., 2012)
Rhabdoviridae	European bat lyssaviruses 1 (Lyssavirus genotype 5)	*Eptesicus serotinus* *Vespertilio murinus*	Europe	Microscopy, isolation PCR	(Fooks et al., 2003) (Bourhy et al., 1992) (Van Der Poel et al., 2005) (Schatz et al., 2013)
		Eptesicus serotinus *Myotis myotis* *Myotis nattererii* *Miniopterus schreibersii* *Rhinolophus ferrumequinum* *Tadarida teniotis* *Vespertilio murinus*	Spain	Microscopy, serology, PCR	(Serra-Cobo et al., 2002)
		Rousettus aegyptiacus	Zoo bat in Denmark imported from the Netherlands	Microscopy, isolation	(Bourhy et al., 1992b)
	European bat lyssaviruses 2 (Lyssavirus genotype 6)	*Myotis daubentonii* *M. dasycneme*	Europe	Microscopy, serology, PCR	(Fooks et al., 2003) (Serra-Cobo et al., 2002) (Schatz et al., 2013)
		Myotis daubentonii	Scotland	Isolation serology, PCR	(Brookes et al., 2005)
		Myotis daubentonii	United Kingdom	Microscopy, isolation PCR	(Johnson et al., 2003)
	Bokeloh bat lyssavirus	*Myotis nattereri*	France	Isolation	(Picard-Meyer et al., 2013)
	Lleida bat lyssavirus	*Miniopterus schreibersii*	Spain	PCR serology	(Aréchiga Ceballos et al., 2013)
	Dimarhabdovirus	*Eptesicus isabellinus*	Spain	PCR	(Vázquez-Morón et al., 2008)

1