Aus dem Institut für Mikrobiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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Astrid Kathrin Nowak
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Mieux vaut tard que jamais.

Summary

Emerging infectious diseases (EIDs) play an increasingly important role for human health. Although most research is focused on the detection of new viral pathogens, 54% of EID's are of bacterial origin, including a large number of drug-resistant types. Tropical regions, especially sub-Saharan Africa, represent 'hotspots' for zoonotic EIDs, as the species richness is particularly high in those areas.

Several features of physiology and ecology make bats unique in their role as reservoir and distributors for pathogens. In the present study, I combine a histopathological approach with micro- and molecular biological techniques in order to investigate the health state of and potential bacterial pathogens carried by asymptomatic free-living fruit bats (*Eidolon helvum, Myonycteris torquata, Rousettus aegyptiacus, Megaloglossus woermanii, Hypsignathus monstrosus*) from central and western Africa.

Histopathological analyses of 131 bats revealed lesions mainly in lungs and livers. Almost one fourth of the bats (24.8%) showed granulomatous liver lesions of different developmental stages. The main lesion of the lungs was lobular interstitial infiltration in 26.9% (n=35) of the animals. Fifty-five animals were randomly chosen for a broad 16S rDNA screening, which gave hints for the presence of extra intestinal pathogenic *Escherichia coli* (ExPEC). Thirty-seven *E. coli* sequence types (STs) were identified including unknown as well as STs frequently linked to infectious diseases. Only few of the STs isolated showed intermediate resistance to certain antimicrobial substances tested. This, together with the mainly arboreal lifestyle of the fruit bats, points towards a rather "naïve" population of *E. coli* in those fruit bats investigated. A full genome analysis of a strain of ST131 revealed that the strain belongs to an older clade, usually not linked to antimicrobial resistance. Given the high number of new STs, further studies are needed to describe the *E. coli* diversity circulating in wildlife, especially in the understudied tropical setting.

Even if not very likely, transmission of ExPEC to humans or domestic animals should be considered, as larger fruit bats, including those species investigated here, are frequently hunted as bushmeat. This results in ample opportunities for transmission, e.g. through preparation and consumption. In addition, several fruit bat species have adapted to human settlements, where they exist in large quantities and contaminate the environment, including food markets, with potentially pathogenic bacterial types.

Zusammenfassung

Afrikanische Flughunde als potentielles Reservoir für zoonotische Krankheitserreger am Beispiel von *Escherichia coli*

Neuartige Infektionskrankheiten stellen eine zunehmende Bedrohung für die menschliche Gesundheit dar. Auch wenn die Entdeckung neuartiger viraler Pathogene im Vordergrund vieler derzeitiger wissenschaftlicher Untersuchungen steht, sind 54% der neuartigen Infektionskrankheiten bakteriellen Ursprungs, darunter eine Vielzahl antimikrobiell-resistenter Keime. Aufgrund der hohen Artenvielfalt gelten tropische Regionen, allen voran Sub-sahara Afrika, als "Hotspots" für neuartige zoonotische Infektionskrankheiten. Fledertiere werden als Reservoir für zahlreiche virale Krankheitserreger in Betracht gezogen. Einige Eigenschaften hinsichtlich ihrer Physiologie und Ökologie machen Fledermäuse einzigartig in ihrer Rolle als Reservoir und Verbreiter von Krankheitserregern.

In der vorliegenden Studie wurden wildlebende Fledermäuse und Flughunde aus Zentral- und Westafrika auf potentiell pathogene bakterielle Erreger hin untersucht. Dabei wurde ein histopathologischer als auch ein mikro- und molekularbiologischer Ansatz gewählt, um Informationen über den Gesundheitsstatus sowie die bakterielle Belastung asymptomatischer Fledermäuse zu gewinnen.

Bei der histopathologischen Untersuchung von 131 Fledermäusen konnten Gewebsveränderungen am häufigsten in Lungen und Lebern festgestellt werden. Beinahe ein Viertel (24.8%) aller untersuchter Fledermäuse zeigte granulomatöse Veränderungen unterschiedlicher Entwicklungsstadien in der Leber. Häufigste Läsion der Lunge war eine lobuläre interstitielle lymphozytäre Infiltration in 26.9% (n=35) aller untersuchter Tiere, überwiegend mit geringgradiger Ausprägung. Fünfundfünfzig zufällig ausgewählte Tiere wurden mittels 16S rDNA PCR auf Bakterien untersucht, was Hinweise auf extra intestinal pathogene *Escherichia coli* (ExPEC) ergab. Im Folgenden wurden isolierte *E. coli* näher charakterisiert, da sie im Zusammenhang mit einer Vielzahl von Krankheitsbildern bei Mensch und Tier stehen.

Siebenunddreißig verschiedene *E. coli* Sequenztypen (STs) wurden identifiziert, darunter sowohl noch unbekannte, als auch STs, die häufig mit Infektionskrankheiten bei Mensch und

Tier in Verbindung gebracht werden. Nur wenige der STs waren intermediär resistent gegenüber wenigen der 15 getesteten antimikrobiellen Substanzen. Gemeinsam mit dem überwiegend arborealen Lebensstil von Flughunden deutet dies auf eine eher "naïve" *E. coli* - Population in den untersuchten Flughunden hin. Die Vollgenomanalyse eines Stammes des ST131 ergab die Zugehörigkeit zu einer phylogenetisch älteren Klade, die gewöhnlich nicht mit antimikrobieller Resistenz in Verbindung gebracht wird. Angesichts der hohen Anzahl an unbekannten Stämmen sollten weitere Studien durchgeführt werden um die *E. coli*-Diversität bei Wildtieren näher zu beschreiben, vor allem in den bisher wenig untersuchten Tropen.
Wenngleich nicht sehr wahrscheinlich, sollte die Übertragung der isolierten ExPEC auf Menschen oder domestizierte Tiere berücksichtigt werden, da Flughunde (einschließlich der hier untersuchten Arten) häufig als bushmeat gejagt werden, was in einer Vielzahl von Übertragungsmöglichkeiten resultiert bspw. bei der Zubereitung oder dem Verzehr der Tiere. Darüber hinaus haben sich einige Flughundarten an menschliche Siedlungen angepasst, wo sie in großen Kolonien leben und die Umwelt kontaminieren können.

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Abbreviations

AMR	Antimicrobial Resistance
GIT	Gastrointestinal tract
EID	Emerging infectious disease
E. coli	*Escherichia coli*
IPEC	Intestinal pathogenic *E. coli*
ExPEC	Extra intestinal pathogenic *E. coli*
EPEC	Enteropathogenic *E. coli*
EHEC	Enterohemorrhagic *E. coli*
STEC	shiga-toxin producing *E. coli*
ST	Sequence type
ESBL	Extended spectrum ß-lactamase
PFGE	Pulsed field gel electrophorese
CLSI	Clinical And Laboratory Standards Institute
CTX-M	Cefotaximase-Munich beta- lactamase
EcoR	*Escherichia coli* Reference Collection
IMT	Institute of Microbiology and Epizootics
Kb	kilobases
µg	microgramm
MLST	Multilocus sequence typing
PAI	Pathogen associated island
PCR	Polymerase chain reaction
MP	Multiplex PCR
ST	Sequence type
UTI	Urinary tract infection

VAG	Virulence associated genes
PNOK	Parc National d'Odzala-Kokoua
IFO	Industrie forestière d'Ouesso
IZW	Institute for zoo and wildlife disease
HE	Haematoxilin Eosin
ZN	Ziehl-Neelsen
BHI	Brain heart infusion
LEE	Locus of enterocyte effacement

1 Introduction

1.1 Emerging infectious diseases

Emerging infectious diseases (EIDs) are defined as diseases which result from newly evolved strains of pathogens (e.g. multi-drug resistant bacteria), from pathogens that have recently entered a population for the first time or from pathogens that have been known for some time and rapidly and recently increased in incidence or geographic range (Daszak, 2000; Jones et al., 2008b).

Over sixty percent of EIDs are caused by zoonotic pathogens, which are pathogens transmitted between humans and animals. The majority of these zoonoses (72%) originate in wildlife harbouring a "zoonotic pool", from which previously unknown pathogens may emerge and which is particularly rich in the tropics. Here, sub-Saharan Africa counts as one of these specific regions with a particularly high spill over risk, a so-called hotspot of EIDs (Jones et al., 2008b). Although viral pathogens (especially RNA viruses) are seen as a major threat to human health, pathogenic bacteria of animal origin clearly have the potential to cause severe infections in humans and/or animals. 54% of EID events are of bacterial origin, including a large number of drug-resistant bacteria (Jones et al., 2008b).

During the last decade, scientists discovered a new type of EIDs: new strains of common bacteria such as *Escherichia coli*, many of which evolved resistance towards one or multiple antimicrobial agents. Certain strains of pathogenic *Escherichia coli* such as for example classical enterohemorrhagic *E. coli* (EHEC) serotype O157: H7 have an animal reservoir mainly in farmed animals/ ruminants. But also other animals like rabbits have been discussed as potential reservoirs for EHEC (García and Fox, 2003; Scaife et al., 2006) and reports of infections with this enteric pathogen come from almost every continent (Spickler et al., 2010). Some strains of *E. coli* developed resistances towards one or multiple antimicrobial agents. Resistances towards extended-spectrum-ß-lactamases (ESBL) are increasing and spreading worldwide (Paterson and Bonomo, 2005; Cantón and Coque, 2006; Hawkey, 2008). Nowadays some *E. coli* sequence types (STs) gain worldwide attention due to their production of ESBLs and a high virulence, resulting in partly severe infections, which become increasingly difficult to treat. In general, resistant bacteria raise great concern as they frequently fail to respond to conventional treatment. An increase in infections with

antimicrobial resistant bacteria is observed not only in industrial countries, but is of particular relevance also in developing countries where treatment beyond the first line medicals is limited due to cost and availability (Gyansa-lutterodt, 2013; Laxminarayan et al., 2014). The impact is particularly high when resistant pathogens enter health care facilities.

Most reports of antimicrobial resistant bacteria come from the field of human or domestic animal medicine, but due to intensified research efforts it could be demonstrated that antimicrobial resistant *E. coli* also commonly occur in wildlife (Gilliver et al., 1999; Sherley et al., 2000; Österblad et al., 2001; Carattoli, 2008; Costa et al., 2008; Guenther et al., 2010a, 2012a). Occurrence of resistances is mainly linked to the use of antimicrobial agents in humans and livestock, but even if human vicinity has been reported to be one of the driving forces for antimicrobial resistance in bacteria (Skurnik et al., 2006), resistant *E. coli* strains have also been found in very remote areas (Wheeler et al., 2012). Deforestation of rain forests, the land use of cleared areas and habitat fragmentation forces wild animals to leave their previous habitats and to inhabit new niches, which are often located in proximity to human settlements (Halpin et al., 2007; Jones et al., 2008a). In parallel, humans encroach into former wildlife territory and are thus exposed to a so far unknown microflora. This displacement of habitats of both, humans and animals, results in an increased overlap between humans and wildlife and its so far less investigated microbes (Goldberg et al., 2007; Rwego et al., 2008; Mickleburgh et al., 2009). Transmission of pathogens between animals and humans is conceivable on several ways. It can occur via indirect transmission of pathogens by vectors (insects) or intermediate hosts (e.g. malaria or several parasites) (Gubler, 2002; Sutherst, 2004). However, the key mode of transmission is the direct exposure to blood or other body fluids (e.g. urine, saliva, faeces) of infected animals. For example, several bat species are known to roost in urban areas where humans are easily exposed to urine or faeces of the animals (Kunz, 1982; Hutson et al., 2001). Further on, the ingestion of un- or insufficiently cooked meat poses a high risk of transmission of new zoonotic pathogens to humans (Wolfe et al., 2005). In many African countries mainly fruit bats, but also insectivorous bat species, constitute a delicacy and are seasonally and systematically hunted and sold on bush meat markets (Hennessey and Rogers, 2008; Mickleburgh et al., 2009; Kamins et al., 2011; Vora et al., 2014). As an example, in Ghana the consumption of the frugivorous bat species *Eidolon helvum* is estimated at 128.000 animals per year with an upward tendency (Kamins et al.,

2011). Foodborne illnesses such as infections with pathogenic *E. coli* or other pathogenic bacteria and viruses can be the result of consumption of contaminated food.

A global distribution of pathogens might be given through the increasing international travel due to global trade or the growing popularity on ecotourism in exotic countries and areas. A prominent example for such a pathogen that managed to jump from its animal origin to the human population and to become self-sustaining would be HIV1 and HIV2, originating in great apes and sooty mangabeys in Africa (Sharp and Hahn, 2011). Another example of pathogens originating in wildlife with dramatic consequences for human health would be Ebolavirus, as seen in the last outbreak in Western Africa (Saéz et al., 2014). Not only viruses but also pathogenic bacteria of animal origin clearly would have the potential to cause severe infections in humans and/or animals.

When searching for wildlife microorganisms which may be a threat to humans, two different strategies can be used: a) use of wildlife mortalities as indicators for the existence of relevant pathogens, as demonstrated by examples of research about pathogens in wild great apes (Anthrax, Ebola, SIV/HIV) or b) focus on hosts with specific features such as possible contact to humans (e.g. through bush meat consumption or an oral-faecal infection route) combined with ideally high-density animals exhibiting an extensive niche overlap with humans such as rodents and bats (Calvignac-Spencer et al., 2012).

Other criteria for the selection of target animals might be known resistance to a variety of diseases or migrating behaviour as it allows the spread of infectious agents over long distances. Bats represent good candidates in terms of the aforementioned criteria letting them move more and more in the scientific focus as potential vectors and/or reservoirs of several EIDs of public health or veterinary concern (Calisher et al., 2006; Wang and Eaton, 2007; Kuzmin et al., 2011; Smith and Wang, 2012). To predict the risk of unknown pathogens it is necessary to know to how many and to which pathogens humans are exposed to and how many of these can successfully cross species barriers. However, infection in the natural or reservoir hosts are often asymptomatic and thus the screening of apparently healthy animals is essential.

1.2 Morphology and Ecology of bats

Bats belong to the order Chiroptera (χείρ - cheir = hand, πτερόν - pteron = wing) which evolved over 52 million years ago (Simmons, 2005a). With more than 1300 species this order builds after rodents the second largest order of mammals and makes up about 20% of all known mammal species (Turmelle and Olival, 2009; Kunz et al., 2011). Chiropteran species are one of the most diverse and geographically dispersed vertebrates on earth and are found all over the world except for the Antarctica and some islands (Wilson and Reeder, 2005; Tsang et al., 2016). The huge diversity within this order is already reflected by the very diverse morphology of different bat species (e.g. body sizes ranging from 2g to 1200g) but also by several behavioural and physiological features, such as their mode of orientation.

Based on phylogenetical analyses, the order Chiroptera is divided into two suborders: Yinpterochiroptera and Yangochiroptera. Yinpterochiroptera include mainly Old World fruit- and nectar-feeding, non-echolocating Pteropodidae and the closely related echolocating *Rhinolophoidae*. Yangochiroptera contain the remaining echolocating bats species (Teeling et al., 2005). Based on morphological criteria bats have also been classified into Megachiroptera (also called megabats or fruit bats) and Microchiroptera (also called microbats) (Nowak, 1994), whose main differences lay in the manner of orientation and the different food sources of these two groups. To simplify matters in the following the latter nomenclature will be used.

The group of microchiropteran bats provides about 80% of all bat species (Simmons, 2005b) and has, according to its enormous biodiversity, a broad range of nutrition sources such as insects, blood, fish, small mammals or frogs (Kunz et al., 2011). Characteristically for microbats is the use of laryngeal echolocation to avoid obstacles and to find food sources at night. Due to the insectivorous diet, bats contribute significantly to the reduction of insects in general and of pests in particular (Kunz et al., 2011).

Within the Megachiroptera, the *Pteropodidae* provide the only family with 46 currently recognized genera and about 186 species, which are distributed in the tropical and subtropical regions of Africa, Asia and Australia (Simmons, 2005b). Megabats mainly feed on fruits, while some species are specialized on nectar and pollen feeding. In contrast to microbats, fruit bats do not use laryngeal echolocation but rely on vision and olfaction for orientation with the exception of *Rousettus aegyptiacus*, which uses both visual orientation and echolocation. Frugivorous and nectarivorous species play an important ecological role as pollinator and

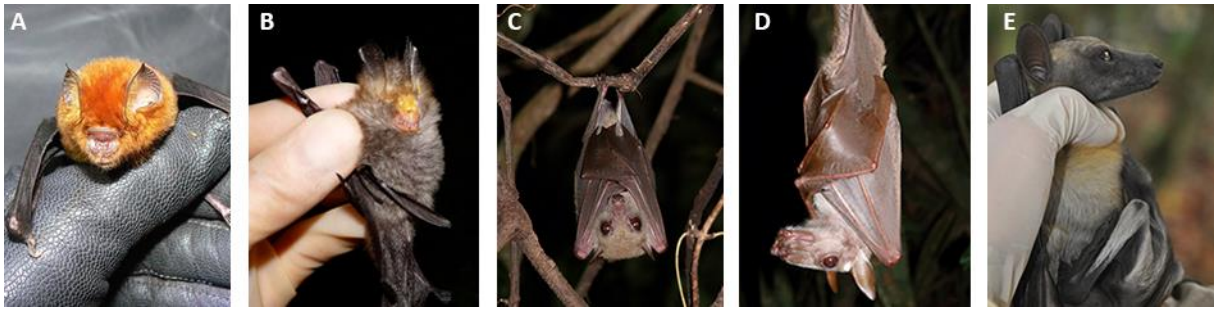
distributor of seeds and have therefore a considerable impact on the economic value of tropical forests (Muscarella and Fleming, 2007; Kelm et al., 2008).

Some special features of morphology, physiology and behaviour make bats quite unique in their role as reservoir and distributors of pathogens. One of the most significant characteristics of this order is the ability of active flight, which is unique among mammals. Some migratory species like *Eidolon helvum* can easily cover distances of more than 2000 km during their seasonal migration (Richter and Cumming, 2008), making them highly suitable to spread pathogens over very long distances. On a more regional level, species like *Hypsignathus monstrosus* fly out every night and can forage in a radius of about 10 to 40 km (Langevin and Barclay, 1990).

Bats show a wide spectrum of different social systems reaching from solitary tree bats (e.g. some small fruit bat species) to colonies of several hundreds of individuals, which enhances the transmission of microorganisms between individuals by bat-to-bat contact. Further on bats inhabit a broad range of ecological niches like caves, tree canopies or hollows in forest and woodland; some species have also adapted to urban areas, where they often roost in man-made structures like crevices, roof trusses or openings in houses. During their yearly migration some species, such as *E. helvum*, often settle in colonies of up to a million and more individuals in trees in dense urban settings leading to frequent human exposure to bat excreta (Plowright et al., 2011).

Due to their extreme longevity of up to 38 years in the wild (Wilkinson and South, 2002) bats can maintain and transmit pathogens to other vertebrates (Calisher et al., 2006) over a long period of time.

Figure 1: Examples of different bat species; Yangochiroptera/ Microchiroptera (A+B) orientate by laryngeal echolocation and are morphologically characterized by comparatively big ears, complex ear and/ or nose creations and small eyes; in contrast Yinpterochiropteran/Megachiropteran species (C, D, E) with big eyes to orientate by vision A) *Hipposideros ruber* B) *Nycteris hispida* C) *Myonycteris torquata* D) *Hypsignathus monstrosus* E) *Eidolon helvum* (Photos K. Nowak)



1.3 Bats as reservoir for zoonotic pathogens

Bats are known to be susceptible to numerous parasitic, fungal, bacterial and viral pathogens (Wibbelt et al., 2007; Jülg et al., 2008; Blehert et al., 2009; Whitaker et al., 2009). First reports of bats being associated with the ecology of human diseases date back to 1911, when rabies virus was identified in bats from South and Central America (Carini, 1911). In the last decades bats could be identified or were suspected as reservoirs of numerous newly discovered highly virulent zoonotic pathogens such as Hendra, Nipah (Halpin et al., 2000, 2011) SARS/Corona-like (Li et al., 2005) and Filoviruses such as Ebola or Marburg viruses (Leroy et al., 2005, 2009; Towner et al., 2007; Pourrut et al., 2009; Saéz et al., 2014).

Compared to the high research effort on viral diseases transmitted by bats, far less is known about bacterial pathogens with zoonotic potential in chiropteran species. Even less data exist on histopathological investigation of bats (Duignan et al., 2003; Mühldorfer et al., 2011b). Due to limitations in sample collection most bacteriological investigations in bats are restricted to the gastrointestinal flora (Klite, 1965; Pinus and Müller, 1980; Heard et al., 1997; Di Bella et al., 2003), serology (Choi and Lee, 1996; Reeves et al., 2006) and bacterial detection by molecular biological methods from blood (Cox et al., 2005; Bessa et al., 2010). Bacteriological examination of faecal, swab - and tissue samples of free living and captive bats revealed 34 different bacteria species in over 60 bat species (Wibbelt et al. 2009), most of which are not obviously associated with disease in humans or other animals. However, some of the identified bacteria are potentially pathogenic to humans, such as *Leptospira* sp (Tulsiani et al., 2011), several enteric pathogens (e.g. *Salmonella* sp*., E. coli*) and also representatives of arthropode-borne bacteria (e.g. *Borrelia*, *Bartonella*). Several enteric pathogens such as *Salmonella* sp., *Shigella* sp. or *Yersinia* sp., including strains, which have been associated with disease in humans have also been detected in different bat species (Brygoo et al., 1971; Moreno et al., 1975; Adesiyun et al., 2009; Reyes, 2011; Mühldorfer et al., 2011b).

Several *Salmonella*-strains, which have been associated with disease in humans and/or animals (Sanchez et al., 2002) were isolated in faeces and tissue samples of both apparently healthy and diseased bats of different micro-and megachiropteran species (Brygoo et al., 1971; Moreno et al., 1975; Reyes, 2011; Mühldorfer et al., 2011b). *Shigella* sp., causative agent of dysenteric infections in humans, has occasionally been isolated from hematophagous and insectivorous bat species in different countries (Arata et al., 1968; Rózalska et al., 1998;

de Souza et al., 2002). *Yersinia* spp. (*Y. pseudotuberculosis* and *Y. enterocolitica*) could be identified as the causative agent for several deaths in captive fruit bats in New York (Williams, 2004; Childs-Sanford et al., 2009) as well as in two free ranging insectivorous bats in Germany (Mühldorfer et al., 2010). Clinical disease in humans is mainly associated with consumption of food or water contaminated with infected animal faeces (Okwori et al., 2009). Flying foxes in Australia revealed high detection rates of pathogenic *Leptospira* sp. in kidney and urine samples (Cox et al., 2005) as well as high seroprevalences (Smythe et al., 2002), indicating that bats might build an important reservoir for this disease. Bats have also been associated with arthropod-borne bacteria such as *Borrelia* sp. (Marinkelle and Grose, 1968; Hanson, 1970; Evans et al., 2009) or *Bartonella* sp., which have been found worldwide in both, micro- and megachiropteran species (Kosoy et al., 2010; Bai et al., 2011; Lin et al., 2012). Other single individual disease incidents identified *Kluyvera* sp. as the cause of death of single bats; however a threat for human health could not be determined (Han et al., 2010). *Pasteurella* sp. and *Pasteurella*-like organisms have occasionally been found as the cause of fatal pneumonia in wild and captive bats, however a zoonotic character of the pathogens identified in those studies has not yet been described (Helmick et al., 2004; Mühldorfer et al., 2011b; Blehert et al., 2014). *Coxiella burnetti*, causing Q-fever in a large number of species has been found in bats in Morocco and southern USSR (Hoar et al., 1998). For an overview of the current state of knowledge concerning the broad spectrum of bacterial pathogens in bats see the review by Mühldorfer (Mühldorfer, 2012).

Escherichia coli has several times been reported to be common in the physiological intestinal flora of micro and megachiropteran bats (Klite, 1965; Moreno et al., 1975; Pinus and Müller, 1980; Heard et al., 1997; Gordon and Cowling, 2003; Adesiyun et al., 2009). Investigations on the detection of *E. coli* are mainly limited to conventional cultivation or identification by PCR, while a more detailed characterization of the isolated *E. coli* strains or determination of the pathogenic potential of isolated strains has only rarely been performed. Few examples are investigations on verocytotoxin producing enterohemorrhagic *E. coli* O157 on few individuals of the species *Carollia perspicillata* and several insectivorous and frugivorous bat species from the Philippines and Brazil (Adesiyun, 1999; Italia et al., 2012; Cabal et al., 2015), but all studies revealed only negative results. Resistances against antimicrobial agents have occasionally been found in *E. coli* originating from bats and other wildlife (Souza et al., 1999; Gopee et al., 2000; Sherley et al., 2000; Costa et al., 2008; Adesiyun et al., 2009; Guenther et

al., 2011). In a comparative study on a broad variety of mammals from Australia and America, *E. coli* isolates from bats had a higher prevalence of antimicrobial resistance than those from other mammals (Souza et al., 1999). Adesiyun et al. (2009) revealed 82% of *E. coli* strains from insectivorous bats being resistant to one or more antimicrobial agents. In a recent study 90% of *E. coli* isolated from bats in Nigeria showed multiple resistances (Oluduro, 2012).

1.4 Escherichia coli

Escherichia coli is a Gram-negative rod shaped bacterium and belongs to the family of Enterobacteriaceae. It typically colonizes the gastrointestinal tract of most warm blooded animals (including humans) and birds within a few hours after birth (Kaper et al., 2004) . Besides those commensal *E. coli*, several strains have acquired specific virulence attributes that allow them to adapt to new niches and cause a broad spectrum of diseases. Virulence factors can be located chromosomally, frequently on so-called pathogen associated islands (PAIs), but many of the virulence associated genes are located on flexible genomic elements such as plasmids, transposons or bacteriophages and can hence easily be transmitted between different bacteria strains by horizontal gene transfer. Several studies have shown that pathogenic *E. coli* strains may be derived from commensal strains by acquiring virulence factors (Tenaillon et al., 2010).

The group of pathogenic *E. coli* can be subdivided into intestinal pathogenic *E. coli* (IPEC), characterised by the possession of distinct combinations of virulence-associated factors, and extraintestinal pathogenic *E. coli* (ExPEC), which possess a broad range of virulence factors.

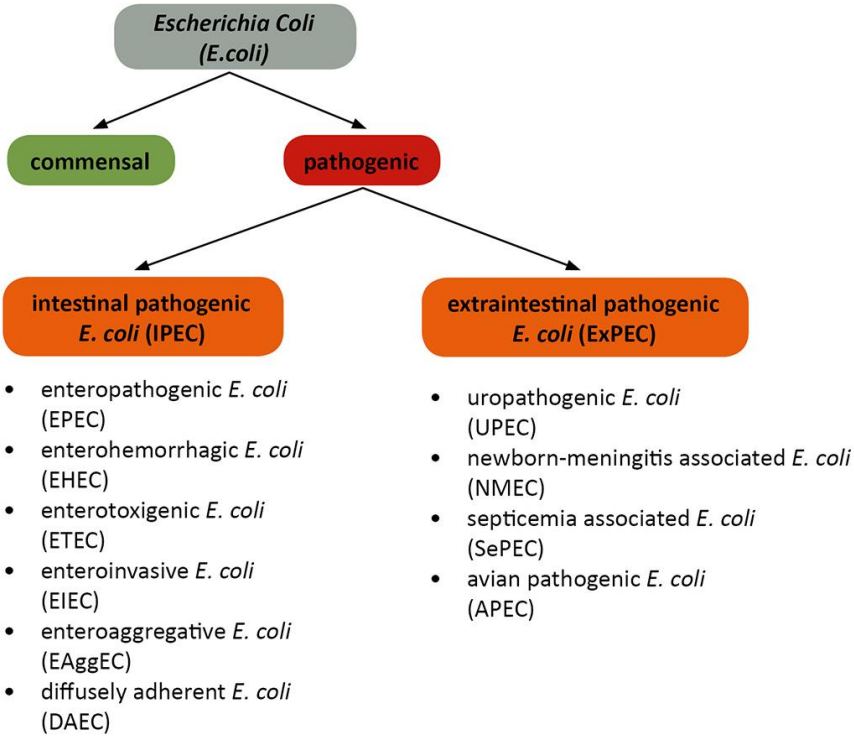
Classical diarrhoeal infections are caused by IPEC, which again can be subdivided into the six pathovars enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EaggEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC), depending on the underlying pathomechanism (Figure 2) (Nataro and Kaper, 1998).

ExPEC can be classified into uropathogenic *E. coli* (UPEC), newborn-meningitis associated *E. coli* (NMEC), septicemia-associated *E. coli* (SePEC) and avian pathogenic *E. coli* (APEC) (Russo and Johnson, 2000) which are the underlying cause for urinary tract infections (UTIs), meningitis or septicaemia respectively. However it could be demonstrated that there are large overlaps between so-called ‘determined’ pathotypes APEC, UPEC and NMEC regarding their

virulence, hindering a clear definition of these pathotypes (Ewers et al., 2007; Tivendale et al., 2010). Besides the mentioned main syndromes ExPEC can also affect almost any other organ and tissue as it can cause intraabdominal infection, pneumonia, osteomyelitis, peritonitis or soft tissue infection (Russo and Johnson, 2003).

ExPEC differ from commensal and intestinal pathogenic *E. coli* phylogenetically and epidemiologically. Phylogenetic analyses have shown that both, intestinal *E. coli* as well as ExPEC strains fall into four main phylogenetic groups (A, B1, B2 and D) (Herzer et al., 1990).

Figure 2: Schematic diagram of *Escherichia coli* groups



Commensal *E. coli* predominantly belong to the phylogenetic group A or B1, most virulence associated genes (VAGs) are lacking (Johnson et al., 2001). Intestinal pathogenic *E. coli* can share the same phylogenetic groups as they derive from A, B1 and D, but harbour specific VAGs which enable a classification to certain pathovars such as *bfp,* encoding for pili, for EPEC (Robins-Browne and Hartland, 2002) or *stx,* which is a defining characteristic of the Shiga toxin-producing *E.coli* (STEC) (Croxen and Finlay, 2010). In contrast, virulent ExPEC mainly belong to phylogroup B2, followed by group D, while group A and B1 are rarely found (Picard et al., 1999; Johnson and Stell, 2000; Johnson et al., 2001). Further on ExPEC

possess a broad range of VAGs that facilitate the movement from the intestinal tract as well as the establishment of infection in extraintestinal organs. Schierack and colleagues claimed that ExPEC seem to have an advantage in the colonization of the intestinal tract of mammals due to certain VAGs (Schierack et al., 2008).

Recognized VAGs that distinguish commensal *E. coli* from ExPEC include diverse adhesins, toxins, siderophores/ iron acquisition, protectins, invasins, serum-resistance systems and factors of miscellaneous or unknown function. All of these factors simplify colonization and invasion of the host, avoidance or disruption of host defence mechanisms and tissue damage. Even if the presence of several putative virulence genes has been positively associated with pathogenicity of ExPEC (Ewers et al., 2007), a categorisation of ExPEC strains into host- or pathotypes just by means of virulence gene typing is not possible. The ability of a bacterium to cause disease is thus not only determined by the pure absence or presence of VAGs or a combination of these; moreover virulence is determined by the level of expression of present VAGs, which can vary between pathogenic and non-pathogenic isolates (Pitout, 2012). Presence of VAGs and the variation in prevalence among different hosts might also reflect a specific adaptation to the host gut flora rather than virulence per se (Tenaillon et al., 2010).

Many of the VAGs are located on mobile genetic elements, such as plasmids, integrons, transposons, or bacteriophages, which can be mobilized and easily transferred via horizontal gene transfer to other bacteria of the same or different species (Allen et al., 2010). Acquiring new traits is essential for the fitness and survival of a pathogen, leading to the continuous evolvement of new strains with new properties.

A worrying development is the increasing number of bacteria resistant towards multiple antimicrobial agents. Particularly ExPEC strains with antimicrobial resistances raise a human health concern, as infections due to members of this group of *E. coli* are difficult or even impossible to treat (Livermore, 2012). One of the most well studied mechanisms of resistance is the production of extended spectrum beta lactamase (ESBL). ESBL is a plasmid located enzyme that leads to resistance towards cefalosporines of the third and fourth generation. Additionally ESBL-producing strains are frequently resistant to other antimicrobial agents such as fluoroquinolones, aminoglycosides, tetracyclines or sulphonamids. Strains possessing ESBL-genes have been shown to contribute to increased virulence (Schaufler et al., 2016).

1.5 Objectives

In the present study, I combine a histopathological approach with micro- and molecular biological techniques in order to investigate the health state of, and potential bacterial pathogens carried by asymptomatic free-living fruit bats (*Eidolon helvum, Myonycteris torquata, Rousettus aegyptiacus, Megaloglossus woermanii, Hypsignathus monstrosus*) from central and western Africa.

The main objectives are:

1) Determination of the health status of chiropteran hosts by histopathology
2) Screen selected chiropteran hosts for bacteria infection by creation of a 16s rDNA molecular bacterial library
3) Characterization of *E. coli* strains detected in chiropteran hosts

2 Material and Methods

A major part of this work was the collection of samples. Not all of the collected samples were used for further analyses. Analytical work can be divided into four sections:

1) Histopathology to investigate morphological changes resulting from the impact of microorganisms on chiropteran hosts
2) Generation of a 16s rDNA library of selected bats with and without histological lesions to detect potential links between pathogens and lesions
3) Cultivation of bacteria from corresponding, mainly intestinal tissue samples
4) Detailed characterization of cultured *E. coli*

Figure 3: Working steps of present study. 131 bats including insectivorous and fruit eating species were histopathologically analysed. 55 animals were randomly chosen for a broad 16S rDNA screening; based on results and availability, 51 samples were cultivated resulting in 41 *E. coli* isolates. Further characterization of the 41 isolates included determination of Virulence associated genes (VAGs), Multilocus sequence typing (MLST), Pulsed field gel electrophoresis (PFGE) and testing for antimicrobial resistance (AMR)

2.1 List of Materials

2.1.1 Equipment

Name	Company
Analytical balance	Sartorius AG, Göttingen
Biological Safety cabinet HeraSafe	Thermo Fisher Scientific Inc., Rockfort, IL, USA
Cell disruptor (FastPrep -24 Instrument)	MP Biomedicals, Heidelberg
Centrifuge EP 5415D, EP5417C	Eppendorf AG, Hamburg
CO_2 Incubator C200	Labotect GmbH, Göttingen
Climbing rope 6mm	Edelrid
Dehydrator Excelsior AS	Thermo Fisher Scientific GmbH, Dreieich
Electrophoresis chambers	AGS; Hybaid; MWG
Tissue Embedding System,Leica EG1150C	Leica Biosystems Nussloch GmbH
FlexCycler	Biozym Scientific GmbH, Hessisch Oldendorf
Gel documentation system	PHASE Gesellschaft für Phorese, Analytik und Separation mbH, Lübeck
Gel electrophoresis chamber	neoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg
Harptrap	Faunatech, Victoria, Australia
Incubator Heraeus B6060	Thermo Fisher Scientific GmbH, Dreieich
Microscope Axioskop	Zeiss, Germany
Microwaves	SB-Großhandels GmbH, Quelle Gruppe, Nürnberg
Mistnets	Reiner Vohwinkel, Germany
Nanodrop	ND-1000 PEQLAB Biotechnologie GmbH, Erlangen
Pipettes	Eppendorf AG, Hamburg
Power supply (Consort EV2.31)	Sigma-Aldrich Chemie GmbH, Steinheim
PowerPac Basic Power Supply	Bio-Rad Laboratories GmbH, München
Precision balance	Sartorius AG, Göttingen
Rotary Microtom, Microm HM340E	Thermo Fisher Scientific GmbH, Dreieich
Sequencer (ABI Prism 3130xl Genetic Analyzer)	Life Technologies GmbH, Darmstadt
Liquid nitrogen tank	Bayern genetics
Vortexer (REAX top)	Heidolph Instruments GmbH & Co.KG, Schwabach
Waterbaths	Julabo; Th. Karow GmbH

2.1.2 Kits

Name	Company
Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit	Life Technologies™ GmbH, Darmstadt
ExoSAP-IT For PCR Product Clean-Up	Affymetrix Inc., Santa Clara, CA, USA
GeneMATRIX Stool DNA Purification Kit	Roboklon, Berlin

Invitrogen ™ Platinum Taq DNA Polymerase	Life Technologies™ GmbH, Darmstadt
Invitrogen ™ Kit for Sequencing with One Shot ® TOP10 Chemically Competent E. coli	Life Technologies™ GmbH, Darmstadt
JETQUICK Gel Extraction Spin Kit	Genomed GmbH, Löhne
Master PureTM Genomic DNA Purification Kit for blood version II	Biozyme Diagnostics GmbH, Hessisch Oldendorf
NucleoSpin®RNA II	Macherey-Nagel GmbH & Co. KG, Düren

2.1.3 Consumables

Name	Company
Agar plates (Columbia, Gassner, Chrom)	Oxoid Deutschland GmbH, Wesel
Antimicrobial plates	Oxoid Deutschland GmbH, Wesel
Api 20E tests	Biomerieux, Nürtingen
Cotton swabs (different sizes, sterile)	Heinz Herenz, Medizinalbedarf GmbH, Hamburg
Cryotubes 1.2ml and 1.8ml	Carl Roth® GmbH & Co. KG, Karlsruhe
Cover glasses	Carl Roth® GmbH & Co. KG, Karlsruhe
Embedding cassetes	Carl Roth® GmbH & Co. KG, Karlsruhe
Forceps (disposable)	Carl Roth® GmbH & Co. KG, Karlsruhe
BHI-broth	Oxoid Deutschland GmbH, Wesel
Microtome blades Mx35 Premier+	Thermo Fisher Scientific GmbH, Dreieich
Microscope slides	Carl Roth® GmbH & Co. KG, Karlsruhe
ParaPlast	Carl Roth® GmbH & Co. KG, Karlsruhe
Parafilm M	BRAND GmbH & Co. KG, Wertheim
Reaction tubes (0.5ml)	Carl Roth® GmbH & Co. KG, Karlsruhe
Reaction tubes (1.5ml, 2ml)	SARSTEDT AG &Co. Nümbrecht
Roti®-Histol	Carl Roth® GmbH & Co. KG, Karlsruhe
Scalpels (disposable)	Carl Roth® GmbH & Co. KG, Karlsruhe
Staining solutions (Hemalaun, Eosin)	Carl Roth® GmbH & Co. KG, Karlsruhe
Surgical gloves (Biogel Diagnostic)	Mölnlycke Healthcare GmbH, Erkrath
TYVEC body suits	Carl Roth® GmbH & Co. KG, Karlsruhe
Whatman paper (GB003)	GE Healthcare, Buckinghamshire, UK

2.1.4 Chemicals

Name	Company
Invitrogen™ 1kb DNA ladder	Life Technologies™ GmbH, Darmstadt
Invitrogen™ 100kb DNA ladder	Life Technologies™ GmbH, Darmstadt
2-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Steinheim
Agar	BD, Heidelberg
Ambion®Nuclease free water	Life Technologies™ GmbH, Darmstadt

Ambion®RNA-later®	Life Technologies™ GmbH, Darmstadt
Blue/Orange loading Dye, 6x	Promega GmbH, Mannheim
Ethanol	Carl Roth® GmbH & Co. KG,Karlsruhe
Formalin 38%	Local pharmacies
Ketamin (50mg/ml)	WDT eG, Garbsen
Rompun®(Xylazin) 2%	Bayer AG, Leverkusen

2.1.5 Software

Name	Company
Adobe Creative Suite (Photoshop CS5)	Adobe Systems Software Ireland Limited, Dublin Ireland
Basecamp	Garmin, Garching, Deutschland
Geneious Pro v5.5.7	Biomatters Ltd., Auckland, New Zealand
Microsoft Office 2010	Microsoft, Redmont, WA, USA
Quantum GIS	QGIS Development Team
RidomSeqSphere 0.9.19	Ridom GmbH, Münster, Deutschland
RAxML 8.1	Heidelberg Institute for theoretical Studies

2.2 Bat trapping and collection of animal samples

Samples were collected in Sierra Leone and the Republic of Congo during three fieldtrips in 2009 and 2010 by the author. Choice of capture sites was based on the history of ebolavirus outbreak in the regions and is not related to this study. To enable a comparison of the composition of species in different kinds of habitat, a broad variety of bats including insectivorous and frugivorous species was captured in primary forest, secondary forest and degraded areas like manioc fields or banana plantations. In this study however those data were not taken into account.

Samples from Sierra Leone were collected from February to April 2009 at ten study sites within the Gola Forest Reserve. In the Republic of Congo bats were collected between August and October 2009 in the Odzala National Park (Parc National d'Odzala-Kokoua (PNOK)) at five sites along the Mambili River in the north west of the country in. In this mission additional living bats from a bush meat market in Brazzaville were sampled.

The third mission was from February to April 2010 in the province of Sangha in the area of the Industrie forestière d'Ouesso (IFO) Logging Concession in the north of the country, south of Ouesso. Authorizations were obtaines as described elsewhere (Nowak et al. 2017).

2.2.1 Field sites

Gola Forest Reserve, Sierra Leone (UTM 29N 250058 815300)

The Gola Forest Reserve is located in the southeast of the Eastern Province of Sierra Leone and comprises the largest continuous forest block of the country. It is part of the Upper Guinea Forests, which ranks as one of the global 25 biological diversity hotspots, comprising 50 mammal species (Golarainforest.org, 2013). The Gola Forest Reserve has a surface of 750km² and is divided into three parts: Gola Central with 460 km², Gola South with 230 km² and Gola North with 60 km² (Fig. 2). Since 2009 the Gola Forest Reserve is part of the Trans-boundary Peace Park, which unites the Gola Forest Reserve in Sierra Leone (75 000 ha) and the Lofa and Foya Forest Reserves in Liberia (80 000 ha and 100 000 ha respectively) (http://www.africa-eu-partnership.org/en/success-stories/trans-boundary-peace-park-sierra-leone-and-liberia, 2013). The predominant vegetation types are moist evergreen and moist semi-deciduous forest. Some areas are lacking large trees due to selective logging (Weber and Fahr, 2009).

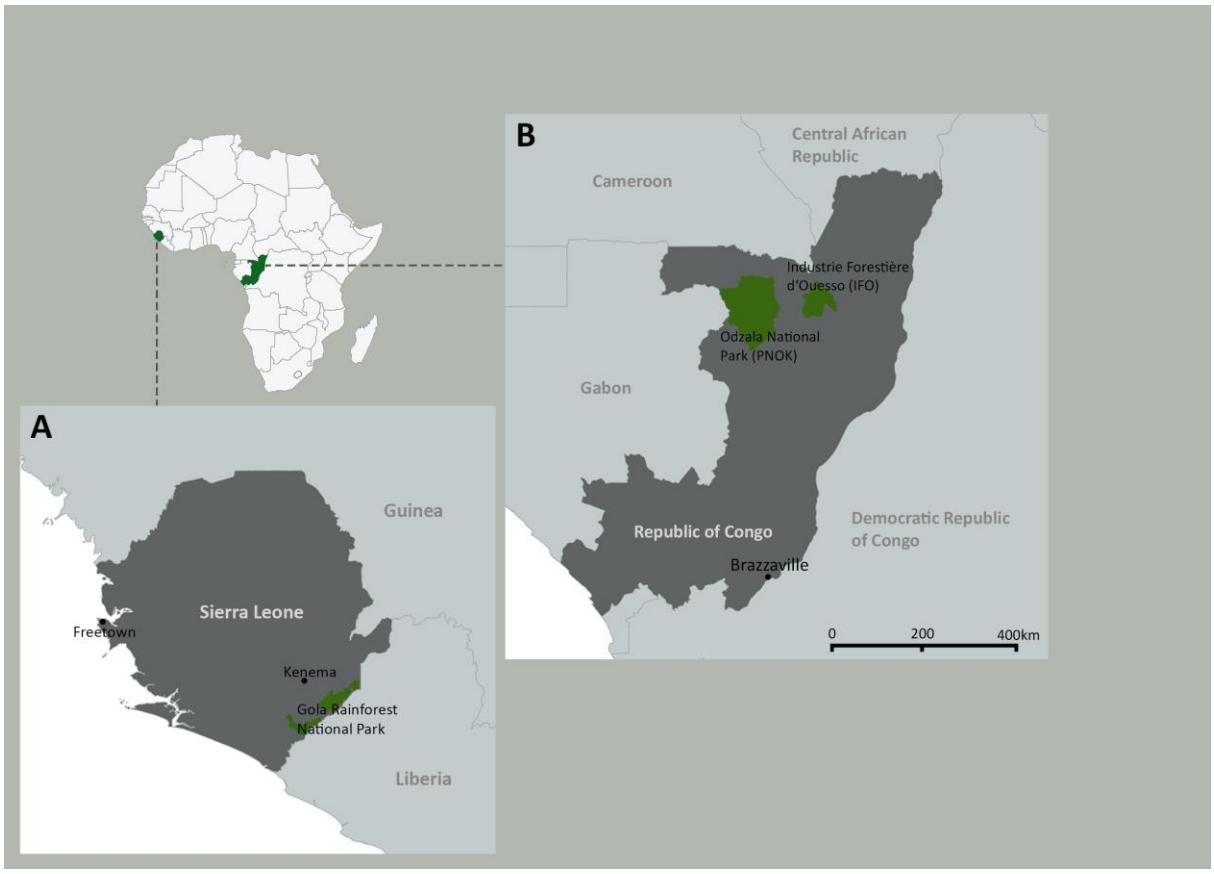
Parc National d'Odzala - Kokoua, Republic of Congo (UTM 33N 466742 48256)

The Parc National d' Odzala – Kokoua (PNOK) is located in the north western part of the Republic of Congo (RC), close to the border to Gabon. Founded in 1935 it is one of the oldest national parks in Africa with a surface of 13600 km². Since 1977 it is declared a UNESCO Man and Biosphere Reserve (unesco.org). The predominant vegetation type is pristine rain forest, interrupted by "bais" or salines, which are swampy, grassy clearings, often visited by forest wildlife. The park provides a habitat for 114 mammal species, including chimpanzee and gorillas.

Industrie Forestière d'Ouesso (UTM 33N 607630 165881)

The Industrie Forestière d'Ouesso (IFO) logging concession is a subsidiary of the Danzer Group. It is located in the north of RC, south of Ouesso and covers an area of nearly 1.16 million hectares (11600km²). Due to selective logging the logging area builds a mosaic of logged and pristine forest. Road constructions throughout the area simplify the access of humans to primary forest and its fauna and thus pose a risk to wildlife. Bat samples were taken in different habitat types (primary forest and secondary forest).

Figure 4: Sample sites in central and western Africa; A) Sierra Leone, Gola Forest with its three parts; B) Republic of Congo with the Odzala National Park and the area of the Logging Concession (Industrie forestière d'Ouesso – IFO)



Bats were captured with 12m and 6m mist nets (Vohwinkel, Germany) at different levels, as different species have different preferences concerning the flight height. To capture insectivorous bats, nets were opportunistically installed near ground level at presumed fly-paths, e.g. along or across rivers. With a rope and pulley system, nets were installed between the canopies at a height of about 15 - 25 m to capture fruit bats, which usually fly rather high. Additionally a harp trap (Faunatech, Victoria, Australia) was used. Bats of Sierra Leone and the Republic of Congo in 2010 had been captured according to a standardized capture procedure including one Canopy net in the centre of the sampling area and eight ground nets around, four of which were placed at new locations every day.

Since the focus of the field session in the Republic of Congo in 2009 was on sampling fruit bats, two canopy nets were installed for opportunistic sampling. Nets were opened before sunset (6 to 6.30 pm) and checked every hour. Closing time depended on the capture rate, but was usually before 1 am. Captured bats were individually put into cotton bags until they were processed. From every individual sex, age, reproductive state, fore arm length and weight was taken.

2.2.2 Sampling

Animal samples included saliva, blood, faeces, urine and tissue.

Blood was taken by venipuncture of the wing vein (V. cephalica) using a 1ml insulin syringe and transferred into a 1.2ml cryotube containing a drop of EDTA (1.5mg Dinatrium-EDTA/ 20µl PBS) to prevent coagulation. If blood volume was too low (e.g. due to low body mass), a drop of blood was dried on filter paper (Whatman, Germany). Saliva was taken with a sterile cotton swab (Heinz Herenz, Germany) from the back of the bats' throat. If possible, faecal and urine samples were taken. Faecal samples were collected from the inside of the cotton bag the bats were transported with or, in cases of spontaneous defecation during examination, excrements were directly transferred into the cryotube. Urine was collected directly into the tube. Parasites (bat flies, ticks, mites) were also collected and preserved in 70 % alcohol, but were not analyzed in this study. The workspace was disinfected with 70% alcohol after every sampling procedure.

The majority of animals were released after sampling. Full necropsies were performed on a limited subset of bats of species known to carry zoonotic pathogens (*Myonycteris torquata, Hypsignathus monstrosus, Epomops franqueti* and *Eidolon helvum*) and on insectivorous bats, which were collected for a reference collection of the University of Ulm. Necropsies were conducted during the day.

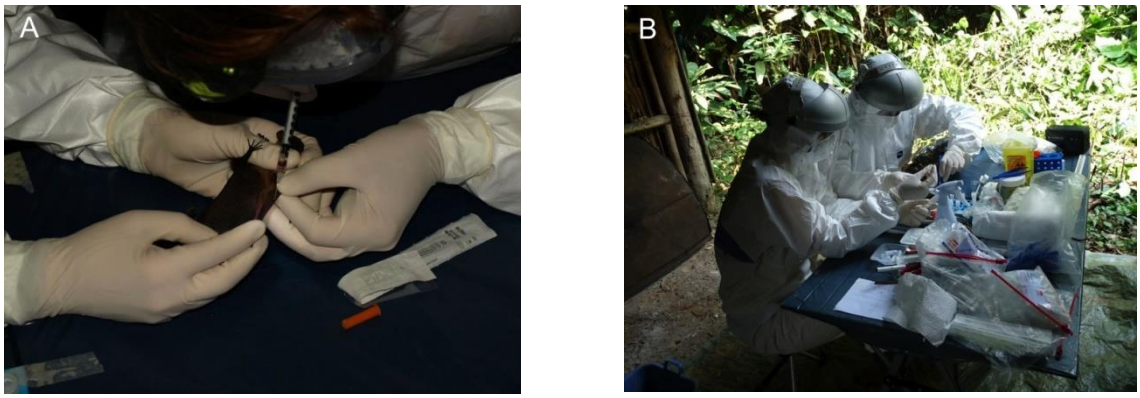
For anaesthesia a mixture of Rompun 2% (Xylazin 20mg/ml) and Ketamin 5% (50mg/ml) (at a ratio of 1:2 and a dose of 0.6ml/ kg (West et al., 2007) was injected intramuscular into the pectoral musculature. For euthanasia animals were bled by cardiac puncture. The study was performed following best practise guidelines for euthanasia in wildlife (Leary et al., 2013) and under authorization of the according wildlife authorities of Sierra Leone and Republic of Congo.

Tissue samples were taken in the order spleen, liver, kidney, lung and intestines and immediately preserved in liquid nitrogen and/ or in RNAlater® (Ambion, Germany). To minimize the risk of contamination the gastrointestinal tract was not opened or damaged until all other organ samples were taken. Additionally, tissue samples were preserved in 10% neutral buffered formalin for histopathological analyses. RNAlater samples were stored at -20°C. Samples conserved in liquid nitrogen were shipped to Germany on dry ice and immediately stored at -80°C upon arrival at the RKI.

Safety measurements

Only trained people conducted capture and handling of animals and samples. Personnel safety equipment for capture included strong leather gloves, goggles and masks. For blood and saliva collection, we wore an apron, goggles, medical facemask and doubled gloves. Necropsies were carried out wearing an all-over-body-suit (Tyvec®, Roth, Germany), FFP 3 safety mask, face shield, arm protection and doubled gloves. Single use disposable scalpels and tweezers were incinerated after use. All other non-disposable equipment was disinfected with potassium 10% hypochlorite. Nets were disinfected every night after use with potassium hypochlorite. The working place and the area around it were covered with thick plastic foil, and to protect it from insects and rain a mosquito net and a tarp were installed. Carcasses of the bats were put into formalin. All rubbish was burned after sampling; residual sharps and carcasses were buried.

Figure 5: Process of bat sampling A) Blood sampling from the wing vein of a *M. torquata* B) Necropsies during the day; PPE included all-over-body-suits, face shields, FFP3 masks and doubled gloves (Photos K. Nowak)



2.3 Histopathology

Spleen, liver, kidney, lung and intestines of 131 individuals were examined histopathologically at the Institute for Zoo- and Wildlife Research (IZW), Berlin under the supervision of Dr. Gudrun Wibbelt. The author personally conducted preparation and microscopical examination of all samples.

Formalin preserved samples were cut and put into embedding cassettes, went through an increasing concentration of alcohol (70%, 80%, 90%, 96%, 99 % Xylen as intermediate) for dehydration and were consecutively embedded into liquid paraffin using an embedding station. Paraffin blocks stayed at -20°C over night. Slices of 3µm were cut with a Rotary

Microtome (Thermo Fisher Scientific GmbH, Dreieich), transferred into a 40°C water bath and put on microscopic slides, which were incubated over night at 60°C.
As a standard colouring all slides were stained with the Hematoxylin-Eosin (HE) staining.
Depending on microscopic findings slices were also stained with Ziehl-Neelson (ZN) staining, Giemsa and Warthin - Starry to detect acid resistant pathogens, parasites or spirochetes respectively.
Slices with accumulation of pigment in the tissue were stained with the Prussian Blue stain after the formalin pigment being bleached with 25% ammonia solution (Kardasewitsch reaction). In single cases slides were stained with von Kossa and PAS stain to detect calcification or glycogen accumulation resp. (protocols of deparaffinisation and staining are listed in the Annex).
All slices were investigated by light-microscopy (Axioskop, Zeiss, Germany) with a 100 to 1000 time magnification.

Table 1: Numbers of histopathologically examined organs from 131 fruit bats from Zentral and Westafrica; numbers of organs vary due to availability of samples; RC= Republic of Congo

Country of Origin (number of sampled bats)	Numbers of organs analysed				
	Liver	Lung	Spleen	Kidney	Intestines
Sierra Leone (n=20)	20	19	12	20	19
RC 2009 (n=60)	58	60	58	58	60
RC 2010 (n=51)	51	51	50	51	51

2.4 Microbiology and molecular biological analyses

Based on the histological findings, 41 livers and 36 lungs of in total 55 animals were screened for a broad range of bacteria using a 16S rDNA PCR. To check whether lesions could be attributed to the presence of certain bacteria, both, individuals showing lesions in lung and/or liver as well as histologically inconspicuous individuals were screened.

2.4.1 DNA Extraction

DNA from frozen lung and liver tissue was extracted with the NucleoSpin® RNA II Tissue Kit (Macherey-Nagel, Düren, Germany). Samples were homogenized with a FastPrep cell

disruptor and further processed following the manufacturer's instructions. DNA was eluted with 60µl nuclease free water and stored at -20°C.

2.4.2 16S rDNA PCR

To test for the presence of bacterial species, liver and lung samples of 55 bats were screened by sequence analysis of the 16S rDNA gene. The 16S rDNA gene encodes for the 16S ribosomal subunit containing both highly conserved sequences, which are common to all bacteria, and divergent sequences unique to each bacterial strain. After extracting DNA from organ tissues using the NucleoSpin RNA II Tissue Kit (Macherey-Nagel, Düren, Germany) the partial 16S rDNA gene was amplified with broadly conserved bacterial 16S rDNA primers D (968f) AACGCGAAGAACCTTACCTG and G (1407r) GACGGGCGGTGTGTAC according to standard PCR protocols (Sambrook et al., 1989) (Table 2).

PCR-products were visualized by gel electrophoresis on a 1.5% agarose gel (1.5g agarose, 66ml TAE-buffer, 34ml GelRed®), running with 120V for 1h 30min. Bands were expected at 450 bp.

Table 2: Protocol and cycling conditions of the 16S rDNA PCR

Mix	[µl]	Cycling conditions	
DNA template	1		
10 x Rxn buffer (without $MgCl_2$)	2.5	94°C 5 min	
$MgCl_2$ (50mM)	2	94°C 30 sec	35x
dNTPs (2,5mM)	0.5	56°C 20 sec	
DFS-Bioron Taq Polymerase	0.075	68°C 40 sec	
Pfu Polymerase	0.1	68°C 7 min	
Primer forward D (967f) (10µl)	0.5		
Primer reverse G (1407r) (10µl)	0.5		
Double distilled water	ad 25		

2.4.3 Cloning, Colony-PCR, Sequence analyses

The 16S rDNA PCR products were cloned into the pCR2.1 vector using the Topo TA cloning kit and chemically competent *E. coli* Top10 cells according to the manufacturer´s instructions

(Invitrogen, Germany). Up to ten clones per transformation were used for sequence analysis. Products older than two hours underwent a refresh PCR to rebuild the Poly-A tail (table 3) before being ligated into the pCR2.1-vector. Three to ten clones per transformation were used for colony PCR and sequence analysis.

PCR products were purified by using ExoSAP-IT (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's instruction and sequenced afterwards. Sequence analysis was performed according to Sanger (Sanger et al., 1977) on an ABI Prism 3100 Genetic analyser using the ABI Big Dye Termination Kit (Table 5). PCR products were sequenced with the same primers that were used during DNA amplification. Cloned sequences were generated with the M13 primers from the Topo TA Cloning Kit. The generated sequences were processed with Geneious 5.3.1 and determined through the Ribosomal Database Project (https://rdp.cme.msu.edu/index.jsp) up to genus level (with a confidence threshold of 95%).

Table 3: Protocol of the refresh-PCR

Mix	[µl]	Cycling conditions
10 x Rxn- Puffer	1.25	10µl mix + 4µl PCR Product
$MgCl_2$	1	70°C 10 min
dNTPs (2.5mM)	1	
Platinum Taq DNA Polymerase	0.125	
Double distilled water	9.38	

Table 4: Protocol of the colony PCR

Mix	[µl]	Cycling conditions	
Sample	1 clone		
10 x Rxn buffer (without MgCl2)	2.5	95°C 5 min	
$MgCl_2$ (50mM)	2	95°C 30 sec	35x
dNTPs (2,5mM)	2	55°C 30 sec	
Primer forward (10pmol)	0.3	68°C 1 min	
Primer reverse (10pmol)	0.3	72°C 7 min	
Platinum Taq DNA polymerase	0.25		
Double distilled water	ad 25		

Table 5: Sequencing protocol

Mix	[µl]	Cycling conditions	
PCR-product	1- 4*	96°C 2 min	
Big Dye	1	96°C 10 sec	25x
5x ABI Buffer	1.5	55°C 5 sec	
Primer (10pmol)	0.5	60°C 4 min	
Double distilled water	ad 10		

* depending on quantity of PCR product

2.4.4 Bacterial cultivation of tissue samples

The 16S rDNA-screening of lung and liver tissue samples indicated the presence of ExPEC in nine individual bats. *E. coli* was chosen for further investigation as it is known to be relevant for human and animal health. *E. coli* has several times been used as an indicator for the directionality of transmission of microorganisms between humans and wildlife (Rwego et al., 2008) and could thus give insights into the human impact on the microbiome of wild animals. Due to this particular interest in determining potentially pathogenic *E. coli* and their antimicrobial resistance phenotype all available organ tissue samples of these nine animals, including lung, liver, kidney, and intestine, were used for cultivation. Moreover, since *E. coli* is frequently present in the intestine and pathogenic members of this species may have their reservoir in the gut (Johnson and Stell, 2001; Johnson and Russo, 2005), the available intestine samples from the remaining animals were also used for cultivation. In total 64 frozen tissue samples from 50 animals (intestines (with faecal content) (n=46), liver (n=6), lung (n=9), and kidney (n=3)) were cultivated.

Thirty-two animals derived from IFO in the north of the RC, another 18 bats came from PNOK in the north western part of the RC, which is almost 200 km distant to the IFO. For five animals cultivation was not performed due to limited sample material (Field-no. 36, 38, 1925, 1953, 2071).

To compensate for sub lethal injuries of the bacteria due to the freezing process at -80°C, all samples were placed in brain heart infusion (BHI) broth and incubated over night at 37°C. Consecutively, the BHI was streaked on Columbia blood agar (5% blood), Gassner agar and Chrom agar (Oxoid, Germany). Identification of bacterial colonies was supervised by Dr. Antina Lübke-Becker at the Institute of Microbiology and Epizootics, Free University Berlin. Purple colonies from Chrom orientation agar were confirmed as *E. coli* by conventional

biochemical tests such as fermentation of glucose and lactose or indole production as described previously (Ewers et al., 2009b). Ability of haemolysis of corresponding colonies was assessed on blood agar. Where biochemical tests revealed ambiguous results, bacterial species were identified with the Api 20E test system (Biomérieux, Germany). One *E. coli* isolate per sample was picked and used for further analyses, except in cases *E. coli* colonies showed two various morphologies on Gassner agar. Then two *E. coli* isolates per sample were taken. All isolates were stored at -80°C in BHI broth with 10% glycerol until further use.

2.4.5 DNA-Extraction

Bacterial DNA from isolates was extracted by using the Master PureTM Genomic DNA-Purification Kit for blood version II (Biozym Diagnostic GmbH, Germany) according to the manufacturer's instruction. DNA was diluted to a working concentration of 50ng/µl.

2.4.6 Virulence genotyping

E. coli isolates were investigated by PCR for 59 genes coding for 59 virulence factors associated with intestinal and extra intestinal pathogenic *E. coli*, following the inhouse procedure developed by the FU Berlin. In detail, targeted genes coded for adhesins (*afa/draBC, bfp, bmaE, csgA, ea-I, eae, fimC, focG, gafD, hra, iha, mat, nfaE, papAH, papC, papEF,papGII/III, sfa/foc, sfaS, tsh*), iron acquisition systems (*chuA, eitA, eitC, feoB, fyuA, ireA, iroN, irp2, iucD, iutA, sitA, sitD* [chromosomal], *sitD* [episomal]), serum resistance (*iss, kpsMTII, neuC, ompA, traT*), toxins (*astA, cnf1/2, hlyA, hlyC, hlyF, sat, stx1, stx2, vat*), invasins (*ibeA, gimB, tia*) and miscellaneous factors (*cvaC, cvi/cva, escV, etsB, etsC, malX, ompT, pic, pks, puvA*).

Primers used in this study were obtained from Sigma Genosys (Steinheim, Germany) and were used in multiplex and single PCR approaches. Primer sequences, including sequences that have been described previously (Yamamoto et al., 1995; Yamamoto and Echeverria, 1996; Schubert et al., 1998; Johnson and Stell, 2000; Runyen-Janecky et al., 2003; Watt et al., 2003; Ewers et al., 2004b, 2007, 2009a, 2010; Rodriguez-Siek et al., 2005; Müller et al., 2006) and references are shown in appendix 1. Additionally genes encoding for enterotoxins (*est-Ia, est-II, eltB-I*) and fimbriae (*fanA, fedA, fasA, faeG, fim41A*) were tested for as

described elsewhere (Franck et al., 1998; Casey and Bosworth, 2009). Positive controls were provided by the IMT Berlin.

The PCR products were visualized by gel electrophoresis with a 1.5% agarose gel and stained with GelRed (7,5g Agarose, 330ml 1xTAE-buffer, 170 GelRed); running conditions were 120V for 1h 30min. Bands/amplicons were photographed at UV exposure.

Table 6: Categories of Virulence associated genes tested by PCR

Function	Gene
Adhesins	*afa/draBC, bfp, bmaE, csgA, ea-I, eae, fimC, focG, gafD, hra, iha, mat, nfaE, papAH, papC, papEF,papGII/III, sfa/foc, sfaS, tsh*
Iron acquisition systems	*chuA, eitA, eitC, feoB, fyuA, ireA, iroN, irp2, iucD, iutA, sitA, sitD* [chromosomal], *sitD* [episomal]
Serum resistance	*iss, kpsMTII, neuC, ompA, traT*
Toxins	*astA, cnf1/2, hlyA, hlyC, hlyF, sat, stx1, stx2, vat*
Invasins	*ibeA, gimB, tia*
Miscellaneous factors	*cvaC, cvi/cva, escV, etsB, etsC, malX, ompT, pic, pks, puvA*

Table 7: Protocol and cycling conditions for Multiplex PCR (MP) I-IX

Mix		**MP I - IV**	**MP V**	**MP VIb/VIc**	**MP VII**	**MP VIII**	**MP IX**
10x Rxn buffer (without $MgCl_2$)		2µl	2µl	2µl	2µl	2µl	2µl
$MgCl_2$ (50mM)		2µl	2.5µl	2.5µl	2.5µl	2.5µl	2µl
dNTPs (10mM)		0.2µl	0.2µl	0.2µl	0.2µl	0.2µl	0.2µl
Primer Mix* (100pmol)		0.6µl	0.3µl	0.6µl	0.2µl	0.25µl	0.4µl
Taq DNA-Polymerase (5U/µl)		0.14µl	0.12µl	0.14µl	0.1µl	0.1µl	0.12µl
DNA Template (50ng/µl)		3µl	3µl	3µl	3µl	3µl	4µl
Double distilled water		ad 20µl	ad 25µl	ad 25µl	ad 25µl	ad 25µl	ad 20µl
Cycling conditions		94°C 3min	94°C 3min	94°C 3min	94°C 3min	94°C 3min	94°C 3min
	25x	94°C 30sec	94°C 30sec	94°C 30sec	94°C 30sec	94°C 30sec	94°C 30sec
		58°C 30sec	57°C 30sec	57°C 30sec	57°C 30sec	57°C 30sec	57°C 30sec
		68°C 3min	68°C 90sec	68°C 90sec	68°C 90sec	72°C 2min	68°C 105sec
		72°C 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min

*mixes for each MP contain primers mentioned in Appendix A.1

2.4.7 Multilocus Sequence Typing

Multilocus sequence typing (MLST) is a technique in molecular biology for the typing of isolates of microbial species by amplifying and sequencing certain slow evolving genes or gene fragments. These so-called housekeeping genes are characteristic for different bacteria. The unique sequences of a given locus are assigned an allele number in order of discovery; combinations of the loci build an allelic profile and are assigned a sequence type (ST). MLST was carried out as described previously (Wirth et al., 2006; Ewers et al., 2010). Gene amplification of the seven housekeeping genes *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase), and *recA* (ATP/GTP binding motif) and sequencing was performed by using primers specified on the *E. coli* MLST web site (http://mlst.warwick.ac.uk/mlst). Sequences were analysed by the software package RidomSeqSphere 0.9.19 (http://www3.ridom.de/seqsphere) and STs were computed automatically.

Table 8: Primers used for Multilocus Sequence Typing

Gene	Description	Primer no.	Lenght	Sequence (5'-3')	Reference
adk	adenylat kinase	3509/ 3510	766bp	s: TCATCATCTGCACTTTCCGC as: CCAGATCAGCGCGAACTTCA	(Tartof et al., 2005)
fumC	fumarase C	2303/ 2691	806bp	s: TCACAGGTCGCCAGCGCTTC as: GTACGCAGCGAAAAAGATTC	(Tartof et al., 2005; Wirth et al., 2006)
gyrB	gyrase B	3747/ 2692	776bp	s: CACCATTCACGCCGATAACT as: ATCAGGCCTTCACGCGCATC	(Wirth et al., 2006; Ewers et al., 2010)
icd	isocitrat dehydrogenase	3722/ 2520	800bp	s: CCGATTATCCCTTACATTGAAG as: GGACGCAGCAGGATCTGTT	(Wirth et al., 2006)
mdh	malat dehydrogenase	3750/ 3751	799bp	s: AGCGCGTTCTGTTCAAATGC as: CAGGTTCAGAACTCTCTCTGT	(Nicolas-Chanoine et al., 2008)
purA	adenylosuccinate dehydrogenase	2307/ 2308	801bp	s: TCGGTAACGGTGTTGTGCTG as: CATACGGTAAGCCACGCAGA	(Tartof et al., 2005)
recA	ATP/GTP binding motif	2694/ 2695	734bp	s: CGCATTCGCTTTACCCTGACC as: TCGTCGAAATCTACGGACCGGA	(Wirth et al., 2006)

Table 9: Protocol and cycling conditions of the PCR utilized for Multilocus Sequence Typing

Mix	[µl]	Cycling conditions	
10x Rxn Buffer (without $MgCl_2$)	3		
$MgCl_2$ (50mM)	1.7	94°C 3min	
dNTPs (2,5mM)	0.15	94°C 30sek	30x
Forward Primer (10pmol)	0.3	56°C 30sek	
Reverse Primer (10pmol)	0.3	72°C 1min	
Platinum Taq Polymerase (5U/µl)	0.08	72°C 10 min	
DNA Template (50ng/µl)	2		
Double distilled water	ad 30		

2.4.8 Whole Genome Sequencing of ST131

E. coli ST131 is an important cause of severe infections including UTIs, respiratory tract infections, wound infections, gingivitis or enteritis (Johnson et al., 2010) and often associated with ESBL-production. This ST gained worldwide attention due to its pandemic dissemination in humans (Tartof et al., 2005; Nicolas-Chanoine et al., 2008; Blanco et al., 2011b; Mora et al., 2011; Alghoribi et al., 2014; Cha et al., 2016) and occasionally in domestic animals and wildlife (Ewers et al., 2012). Due to the high health relevance of this pandemic clone whole-genome sequencing was performed on this strain. DNA was sequenced using an Illumina MiSeq using 300bp paired end reads and 100-fold coverage as described previously (Schaufler et al., 2015). To perform sequence analyses for phylogenetic relationship, sequence data was assembled de novo using CLC Genomics Workbench v 8 (Quiagen). Sequences obtained were compared to published full genomes (Petty et al., 2014) and a phylogenetic tree was generated with RAxML 8.1.Whole Genome Sequencing was performed by Torsten Semmler, RKI.

2.4.9 Pulsed field gel-electrophoresis

Pulsed field gel-electrophoresis (PFGE) was developed by Schwartz and Cantor in 1984 (Schwartz and Cantor, 1984) as a way to separate large DNA fragments. PFGE raises the upper size limit of DNA separation in agarose from 30-50 kb to well over 10 Mb.

PFGE was applied to reveal the clonal relatedness among *E. coli* isolates from different organs from one animal to exclude copy strains or from isolates assigned to the same STs but isolated from different individuals (ST69 (IMT25439, IMT26129, IMT26131, IMT26156), ST127 (IMT26100, IMT25442), ST1146 (IMT26194, IMT26199, IMT26399), ST3225 (IMT25529, IMT25886, IMT25443, IMT25882)).

Isolates were incubated overnight in LB-Medium (lysogeny-broth) at 37°C and adjusted to an optical density OD_{600}=1. Strain IMT 28 served as reference strain. One and a half millilitres of the culture was used for DNA preparation and centrifuged for 5minutes at 9000 rpm to obtain a pellet; the supernatant was discarded. The pellet was resuspended in 1ml of PBS and centrifuged for 5 min. at 9000 rpm and resuspended in 500µl PBS; 250µl were discarded. Samples were warmed to 37°C. The 1.2% agarose gel was melted and warmed to 56°C. Three-hundred and seventy-five microlitres were added to the samples and mixed carefully. Hundred microliters of the mixture were filled into plug moulds and let cool for 15 minutes. Solidified plugs were incubated in 0.5ml ESP-Buffer (containing 0.9mg proteinase K) over night at 56°C. The plugs were washed with 1ml TE-buffer four times for 45minutes each. Plugs were halved and stored in 1x TE-buffer until further use. Before the digestion half a plug was equilibrated in 150µl of 1xTango-buffer. After 30min at room temperature the buffer was removed, replaced with 150µl of fresh Tango-buffer containing 2µl Xbal restriction enzyme. The sample was incubated at 37°C overnight. After digestion buffer and enzyme were removed and the plug was washed with 0.5x TE-buffer. Fragments were separated in a 1.2% agarose gel in 0.5x TBE buffer by using a Chef-DR III Pulsed Field Electrophoresis System (Biorad). Running conditions were 6Volts/cm at 14°C for 20.5 hours. Ramping times were 5-50seconds. Gels were stained with ethidiumbromid and photographed under UV-exposure. PFGE profiles were compared with the unweighted-pair group method using the average linkage method, and Dice similarity indices were calculated (complete linkage; optimization, 1%; position tolerance, 1.5%), using BioNumerics software (version 6.6; Applied Maths, Belgium) (Ewers et al., 2004a).

2.4.10 Phylotype analysis

Determining the phylogenetic membership of an unknown strain already offers important information about its potential pathogenicity. To define phylogenetic groups, we analysed the population structure of *E. coli* by applying a Bayesian approach to estimate global ancestry by sampling from the posterior distribution over global ancestry parameters on the sequences of the seven gene fragments used for MLST with STRUCTURE (Pritchard et al., 2000; Falush et al., 2003). This resulted in the partitioning into six distinct groups. According to Wirth et al. the groups were assigned to four main phylogenetic groups A, B1, B2, and D and two hybrid groups AxB1 and ABD (Wirth et al., 2006). eBURSTV2 analysis (http://eburst.mlst.net/9.asp) was performed to identify clonal complexes (CCs), defined as groups of two or more independent isolates sharing identical alleles at six or more loci.
To reveal the phylogenetic relatedness of the STs identified in the present study to known phylogenetic lineages we compared them to known representatives of ExPEC (ST62, ST73, ST95), EPEC /aEPEC (e.g. ST15, ST20, ST28, and ST29) and commensal strains (ST10) (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) (Figure 12). In order to sort the position of the *Escherichia albertii* strain ST3227, known members of the cryptic *Escherichia* lineages (isolates Z205 from a parrot [ST125], RL325/96 from a dog [ST133] and E10083 [ST546] from a human) (Wirth et al., 2006; Sankar et al., 2009) and of species *E. albertii* were included in the alignment for comparative purposes. eBURST analyses were performed by Christa Ewers, JLU Gießen.

2.4.11 Antimicrobial Resistance Test

Susceptibility against 15 antimicrobials was tested by agar disc diffusion test according to the standards of the Clinical and Laboratory Standards Institute (CLSI, 2013, 2015).
Bacterial material was suspended in Mueller-Hinton broth and incubated up to a density of McFarland 0.5 following the CLSI recommendations. Consecutively the bouillon was plated on the surface of Mueller-Hinton agar. Antimicrobial plates containing amikacin (30 µg), amoxicillin/clavulanic acid (20/10µg), ampicillin (10µg), cefalexin (30µg), cefazolin (30µg), cefovecin (30µg), chloramphenicol (30µg), doxycyclin (30µg), enrofloxacin (5µg), gentamicin (10µg), marbofloxacin (5µg), penicillin (10U), polymyxin B (300 IU),

sulfamethoxazole/trimethoprime (23.75/1.25 µg) and tetracycline (30µg) (BD, Heidelberg, Germany) were placed by hand or using a disc dispenser. Plates were incubated over night at 37°C and the isolates were classified as susceptible, intermediate or resistant according to the breakpoints defined by the CLSI (CLSI, 2013, 2015).

A rapid screening was performed on all isolates to check for ESBL-production. The isolates were plated on Chromagar containing the third generation cephalosporine Cefotaxim (4µg/ml) and incubated over night at 37°C. ESBL-producing strains should show colony growth. *E.coli* isolates were additionally tested for phenotypic ESBL-production with the ESBL confirmatory test recommended by the CLSI, using cefotaxim (30µg), cefotaxim/clavulanic acid (30µg/10µg), and ceftazidim (30µg), ceftazidim/clavulanic acid (30µg/10µg) (CLSI, 2015).

3 Results

3.1 Animal trapping

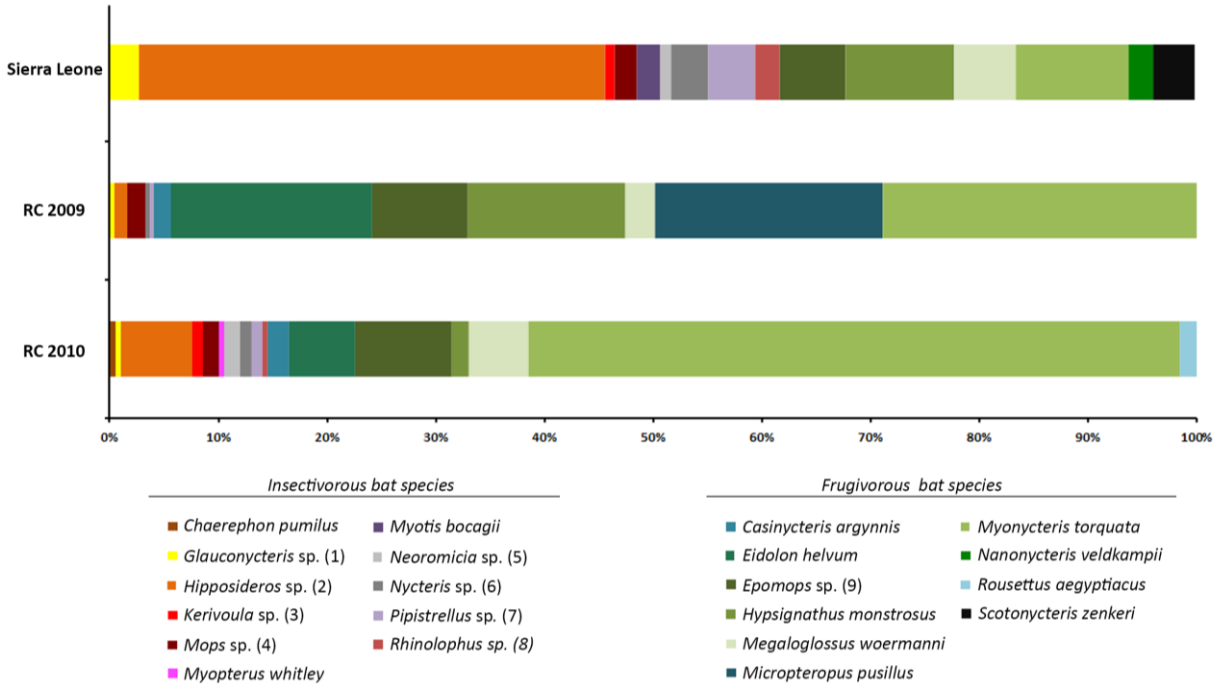
During one field session in Sierra Leone (n=230) and two sessions in the Republic of Congo (RC) (n=203; n=200) a total of 633 bats belonging to 21 different species were captured.
The composition of species in the Gola Forest in Sierra Leone was six frugivorous and nine insectivorous species, whereby the majority of individuals (n=142) were microchiropteran bats (Figure 6). The 203 animals captured in RC in 2009 could be grouped into 13 different species including six frugivorous bat species, which built 77.5% of all animals captured during this field trip. The focus of this field session was on capturing fruit bats, thus the numbers shown in fig. 1 represent the number of sampled bats but don't reflect the whole diversity of bats within the study site. An additional 46 living bats of the species *Eidolon helvum* were bought alive from bush meat hunters on a market in Brazzaville, RC. In the second field session in RC in 2010, 281 bats were captured, but due to logistical reasons samples of only 200 individuals belonging to seven megachiropteran and 16 microchiropteran species were exported to Germany. As in the preceding field session the sampling focus laid on fruit bats.

Table 9: Number (%) of species captured during three field sessions;
RC= Republic of Congo, cf = confer; aff.= affinis, *= animals bought from bush meat hunters

Genus	Species	Sierra Leone N=230	RC 2009 N=249	RC 2010 N=200
Casinycteris	*argynnis*	-	4 (1.6%)	4 (2%)
Chaerephon	*pumilus*	-	-	1 (0.5%)
Eidolon	*helvum*	-	46 (18.5%)*	12 (6%)
Epomops	*buettikoferi*	14 (6.1%)	-	-
Epomops	*franqueti*	-	22 (8.8%)	18 (9%)
Glauconycteris	*beatrix*	3 (1.3%)	1 (0.4%)	1 (0.5%)
Glauconycteris	*cf. poensis*	2 (0.9%)	-	-
Glauconycteris	*cf. variegata*	1 (0.4%)	-	-
Hipposideros	*abae*	3 (1.3%)	-	-
Hipposideros	*beatus*	10 (4.3%)	-	7 (3.5%)
Hipposideros	*cf. caffer*	15 (6.5%)	-	-
Hipposideros	*cf. fuliginosus*	12 (5.2%)	-	1 (0.5%)
Hipposideros	*cf. ruber*	44 (19.1%)	-	4 (2.0%)
Hipposideros	*cyclops*	3 (1.3%)	1 (0.4%)	1 (0.5%)
Hipposideros	*gigas*	2 (0.9%)	1 (0.4%)	-

Genus	Species	Sierra Leone	RC 2009	RC 2010
Hipposideros	*marisae*	4 (1.7%)	-	-
Hipposideros	*spp.*	6 (2.6%)	1 (0.4%)	-
Hypsignathus	*monstrosus*	23 (10.0%)	36 (14.5%)	3 (1.5%)
Kerivoula	*cuprosa*	1 (0.4%)	-	-
Kerivoula	*phalaena*	1 (0.4%)	-	1 (0.5%)
Kerivoula	*smithii*	-	-	1 (0.5%)
Megaloglossus	*woermanni*	13 (5.7%)	7 (2.8%)	11 (5.5%)
Micropteropus	*pusillus*	-	52 (20.9%)	-
Mops	*cf. condylurus*	1 (0.4%)	-	-
Mops	*cf. thersites*	4 (1.7%)	-	3 (1.5%)
Mops	*spp.*	-	4 (1.6%)	-
Myonycteris	*torquata*	24 (10.4%)	72 (28.9%)	120 (60.0%)
Myopterus	*whitleyi*	-	-	1 (0.5%)
Myotis	*bocagii*	5 (2.2%)	-	-
Nanonycteris	*veldkampii*	5 (2.2%)	-	-
Neoromicia	*brunneus*	2 (0.9%)	-	1 (0.5%)
Neoromicia	*nana*	-	-	2 (1.0%)
Nycteris	*arge*	2 (0.9%)	-	-
Nycteris	*grandis*	5 (2.2%)	-	-
Nycteris	*hispida*	1 (0.4%)	1 (0.4%)	2 (1.0%)
Pipistrellus	*aff. grandidieri*	1 (0.4%)	-	-
Pipistrellus	*bellieri*	3 (1.3%)	-	-
Pipistrellus	*crassulus*	-	-	1 (0.5%)
Pipistrellus	*cf. grandidieri*	1 (0.4%)	-	-
Pipistrellus	*nanulus*	5 (2.2%)	1 (0.4%)	1 (0.5%)
Rhinolophus	*alcyone*	-	-	1 (0.5%)
Rhinolophus	*cf. hillorum*	5 (2.2%)	-	-
Rousettus	*aegyptiacus*	-	-	3 (1.5%)
Scotonycteris	*zenkeri*	9 (3.9%)	-	-

Figure 6: Distribution of bat species captured during the study period (Feb-April 2009. August-Oktober 2009, Feb-April 2010) (n=679); RC=Republic of Congo; (1) *G. beatrix; G. cf. poensis; G. cf. variegata* (2) *H. abae; H. beatus; H. cf. caffer; H. cf. fuliginosus; H. cf. ruber; H. cyclops; H. gigas; H. marisae* (3) K. *cuprosa; K. phalaena; K. smithii* (4) *M. cf. condylurus; M. cf. thersites* (5) *N. brunneus; N. nana* (6) *N. arge; N. grandis; N. hispida* (7) *P. aff. grandidieri; P. bellieri; P. crassulus; P. cf. grandidieri; P. nanulus* (8) *R. alcyone; R. cf. hillorum* (9) *E. buettikoferi; E. franqueti*



3.2 Histopathological findings

A subset of 131 individuals of the captured bats were euthanized according to animal welfare guidelines (Leary et al., 2013) and in accordance with the local authorities of Sierra Leone and Republic of Congo. Further macroscopic and microscopic evaluation of tissue samples revealed morphological changes mainly in lungs and livers. Gross findings were irregularly distributed white spots of pin to pinhead size within the liver capsule extending into the parenchyma in four animals (Figure 7A) and enlargement of spleen with activation of lymph follicles in 22.5% of bats (n=27).

Almost one fourth of the bats (24.8%) showed granulomatous liver lesions of different developmental stages, among them two animals where lesions were only visible macroscopically but not microscopically (see above). Granulomas were characterized by a central colliquative necrosis consisting of neutrophils, lymphoid cells and macrophages, surrounded by numerous multinucleated giant cells of foreign body type. In some cases giant

cells were already present in very early stages of development, marked by fresh haemorrhage, so that acute and chronic events could be observed alongside in the same liver.

Despite the typical appearance of a parasitic granuloma no parasite residuals were visible, except for three animals where finely granulated structures suggestive to be of parasitic origin were found in the sinusoids without causing an immunological reaction. Granulomatous liver lesions were only observed in bats of the species *M. torquata* and *H. monstrosus*, but in none of the animals bought from the bushmeat market. A mild to severe vacuolar alteration of hepatocytes could be revealed in 35.7% (n=46) of all investigated livers.

Table 10: Main histological findings in organs of bats from Central- and Westafrica (single case findings or minor changes are summarized as miscellaneous) RC= Republic of Congo; mod = moderate; sev = severe

	Sierra Leone	**RC 2009**	**RC 2010**	**Total**
Liver	N=20	N=58	N=51	N=129
granuloma	-	20 (34.5%)	12 (23.5%)	32 (24.8%)
fatty change of hepatocytes	7 (35%)	18 (31%)	21 (41.2%)	46 (35.7%)
miscellaneous[1]	10 (50%)	13 (22.4%)	8 (15.7%)	31 (24%)
Lung	N=19	N=60	N=51	N=130
beginning mononuclear infiltration	1 (5.3%)	6 (10%)	6 (11.8%)	13 (10%)
mononuclear infiltration (mild - sev.)	4 (21.1%)	18 (30%)	13 (25.5%)	35 (26.9%)
miscellaneous[2]	-	2 (3.3%)	2 (3.9%)	4 (3.1%)
Spleen	N=12	N=58	N=50	N=120
follicular hyperplasia (mild - mod.)	9 (75%)	37 (63.8%)	38 (76%)	84 (70%)
follicular hyperplasia (mod. - sev.)	-	5 (8.6%)	1 (2%)	6 (5.6%)*
hemosiderosis	-	27 (46.5%)	17 (34%)	44 (40.7%)*
Kidney	N=20	N=59	N=51	N=130
protozoal structures	3 (15%)	-	1 (2%)	4 (3.1%)
miscellaneous[3]	-	8 (13.6%)	2 (3.9%)	10 (7.7%)
Intestines	N=19	N=60	N=51	N=112
activated lymphatic tissue	1 (5.2%)	5 (8.3%)	4 (7.8%)	10 (7.7%)
miscellaneous[4]	1 (5.2%)	5 (8.3%)	6 (11.8%)	12 (9.2%)

[1] bile duct hyperplasia, bile duct associated accumulation of macrophages and lymphoid cells, intracellular deposition of pigment, increased number of Kupffer cells, intrasinuosidal parasitic structures, venous thrombus

[2] deposition of pigment, granulomatous lesions

[3] dilatation of tubuli, proliferation of tubular epithelial cells, focal interstitial accumulation of lymphoid cells

[4] helminth residuals, mononuclear and neutrophilic infiltration of lamina propria

* Referring only to Congolese bats

Lymphoid follicular activation of the spleen was macroscopically observed in 22.5% (n=27) of the animals. Mild to moderate activation of the lymphoreticular tissue was seen in 70% (n=84) of the bats, in 5% (n=6) of the spleens follicular hyperplasia was severe. One fourth (n=30) of all spleens appeared histopathologically inconspicuous or had only minimal evidence of reactive follicular hyperplasia.

Hemosiderin deposition within the macrophages of the spleen was observed in 40.7% (n=44) of the Congolese but none of the Sierra Leonean bats. The degree ranged from minimal or mild in 35 animals to moderate in nine bats.

Sixty percent of all lungs (n=78) were histopathologically inconspicuous. The main lesion of the lungs was lobular interstitial pneumonia, characterized by mixed neutrophilic and mononuclear infiltration of alveolar septa in 26.9% (n=35) of the animals; moderate and severe manifestation was only seen in two animals. Lungs of thirteen bats (10%) showed a lobular slightly increased number of neutrophils and macrophages within the interstitium (preliminary stage of pneumonia?). Two animals revealed granulomatous pulmonary lesions surrounded by a capsule of collagenous connective tissue, most likely caused by parasites. Both animals belonged to the species *Eidolon helvum*.

Intestines were striking by the almost complete absence of immunocytes in the lamina propria. Only seven animals revealed a mild or moderate infiltration of neutrophils, lymphocytes and plasma cells in the lamina propria of the small intestines. Hyperplasia of lymphatic structures such as Peyer's patches, the gut associated lymphatic tissue (GALT) and lymphoid follicles was observed in 12 individuals (9.2%). Residuals of nematodes were found in six bats (4.6%), five of them were located intraluminal, one in the interstitium of the villi. Except for one *Hypsignathus monstrosus*, all helminths were found in insectivorous species.

The majority of kidneys (89.2%; n=116) were histologically unremarkable. Four animals had protozoal structures localized either in tubular epithelial cells or in the interstitium. Mild to severe proliferation of tubular epithelial cells was observed in seven animals (5.4%).

Figure 7: Granulomatous liver lesions; A) multifocal white spots on the liver surface (→); B) granulomatous liver lesion; central colliquative necrosis (*) detritus consisting of neutrophils, lymphoid cells and few macrophages, numerous giant cells (→) (HE stain)

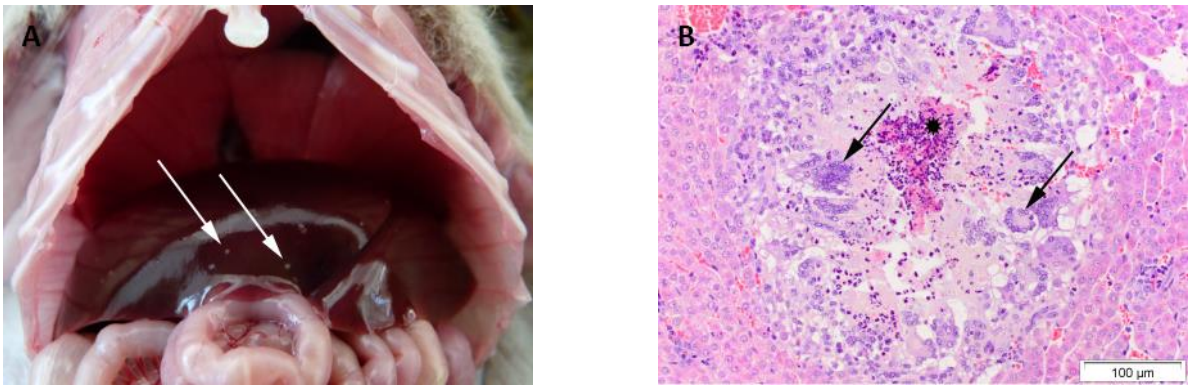


Figure 8: Alterations in lung tissue; A) healthy lung tissue with thin alveolar walls; B) moderate interstitial pneumonia with neutrophilic and mononuclear infiltration of alveolar septa (HE stain)

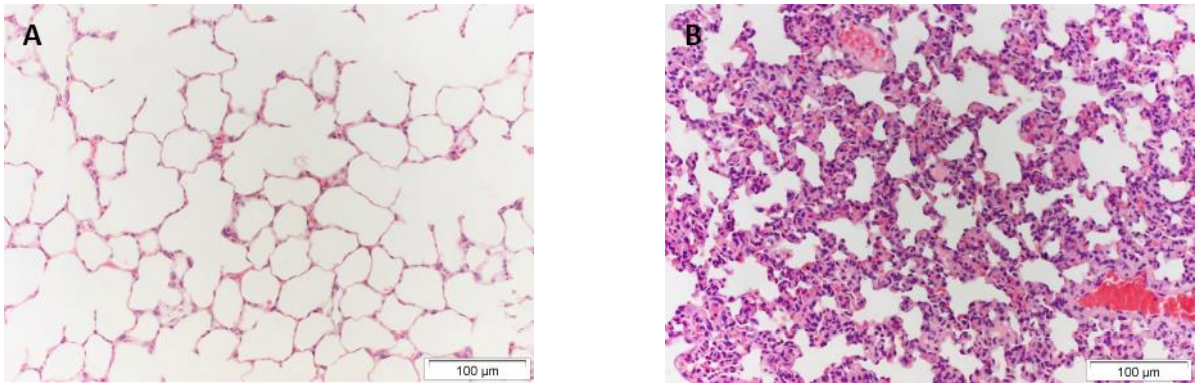


Figure 9: Small intestines, activated Peyer's patches (HE stain)

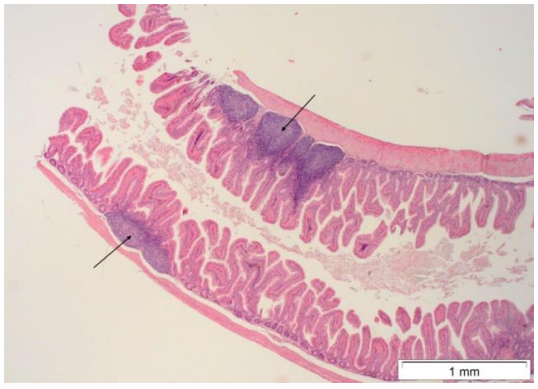
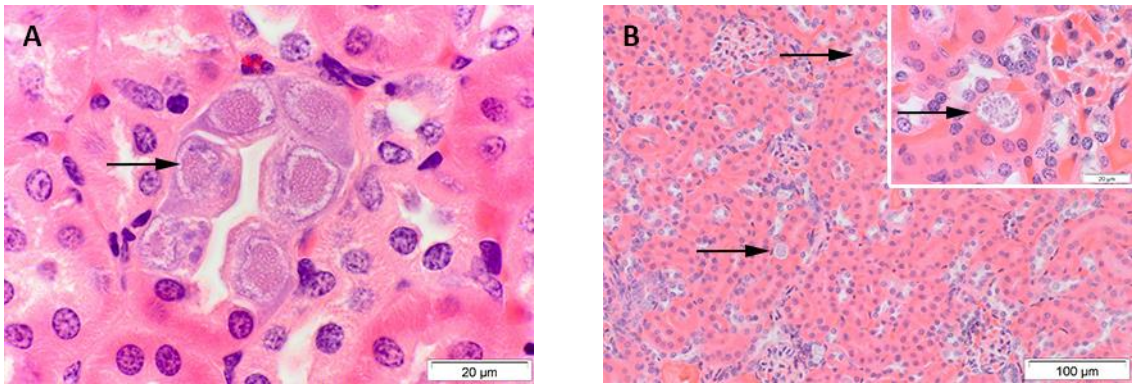


Figure 10: Alteration in kidney tissue; protozoic structures A) in tubular epithelial cells and B) in the interstitium (HE stain)



3.3 16S rDNA PCR

Different organs (liver, lung, spleen, intestines) of 55 fruit bats were molecular biologically examined. Samples were chosen randomly and included organs with and without histological alterations. Sequence analysis of 440-bp 16S rDNA gene fragments indicated the presence of various gram- positive and gram-negative bacteria in the lung and liver tissue samples of 55 African fruit bats (Table 12). Sequences were identified up to genus level by using the online program RDP (Ribosomal Database Project). The confidence threshold was 95%. In several cases identification was only possible up to family level and/or revealed bacteria irrelevant for the purpose of this study. Those are not listed in the following table, except for Enterobacteriaceae and Pasteurellaceae potentially containing representatives relevant for human or animal health.

Predominantly detected bacteria genera were *Acinetobacter* spp. in 74.5% (n=41), *Propionibacterium* sp. in 38.2% (n=21) and *Pseudomonas* sp. in 23.6% (n=13) of all animals examined, regardless whether organs showed histopathological lesions or not. Members of the family Enterobacteriaceae were present in 56.4% (n=31) of all individuals tested. In nine animals, *E. coli* was identified either in the lung or liver sample, and members of Streptococcaceae in 16.4% (n=9) and of Pasteurellaceae in 14.5% (n=8), only one of which could be further identified as *Actinobacillus* sp.. The seven remaining bacteria identified as members of the family of *Pasteurellaceae* could not be confirmed as *Pasteurella spp*. by specific PCR.

Table 11: Bacteria detected in organs of African fruit bats by 16S rDNA PCR

Bacteria	n	Bacteria	n
Gram negative		**Gram positive**	
Enterobacteriaceae		**Streptococcaceae**	
Serratia sp.	8	*Enterococcus* sp.	2
Klebsiella sp.	2	*Lactococcus* sp.	3
Escherichia sp.	9	*Streptococcus* sp.	7
Enterobacter sp.	5		
Citrobacter sp.	1	**Other gram positive**	
Salmonella sp.	1	*Corynebacterium* sp.	3
Enterobacteriaceae[a]	5	*Propionibacterium sp.*	21
		Clostridium sp.	1
Pasteurellaceae		*Bacillus* sp.	1
Actinobacillus sp.	1	*Dietzia* sp.	4
Pasteurellaceae[a]	7	*Paenibacillus* sp.	1
Other gram negative		**Cell wall deficient**	
Pseudomonas sp.	13	*Mycoplasma* sp.	1
Acinetobacter sp.	41		
Bartonella sp.	2		
Ralstonia sp.	4		
Moraxella sp.	1		
Neisseria sp.	1		

[a] identified up to family level

3.4 Bacterial cultivation

Cultivation of 64 frozen intestinal and tissue samples from 50 animals revealed mainly members of the family of Enterobacteriaceae. *E. coli* was the most frequent bacterial species detected in 60% (n=30, 49 isolates) of animals tested. One isolate was identified as *Escherichia albertii* (Table 13). In general, only one *Escherichia* spp. colony per organ sample was further processed, except for cases, where morphological differences could be observed on Gassner Agar (rough and smooth colony surface). Four *E. coli* isolates (12.9%) showed α-hemolysis on blood agar, one isolate was lactose negative. For bacteria others than *E. coli* further characterization was not provided, and those were therefore identified only up to genus level. Other bacteria identified were *Enterobacter spp*. in 23.5% (n=12) and *Klebsiella spp*. in 17.6% (n=9) of animals tested. *Salmonella sp*., *Citrobacter sp*. and *Proteus sp*. were present in one individual each (2%). Representatives of gram-positive bacteria were *Enterococcus spp*. in 51% (n=26), *Staphylococcus spp*. in 11.7% (n=6) and *Bacillus sp*. in one sample (2%).

Table 12: Occurrence of *E. coli* in different African bat species

Bat species	positive for *E. coli* (n)/ animals tested (n)	*E. coli* strains(n)/ isolates (n)
Eidolon helvum	2/ 2	3/ 4
Epomops franqueti	2/ 3	2/ 2
Hypsignathus monstrosus	2/ 2	2/ 2
Myonycteris torquata	22/ 40	31/ 40*
Rousettus aegyptiacus	2/ 3	2/ 2
Total	**30/ 50**	**40/ 50**

* including one *E. albertii*

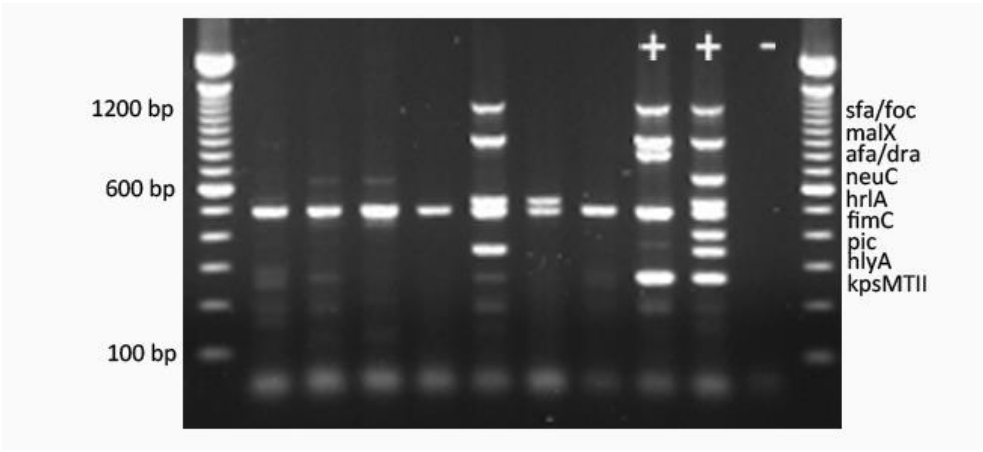
3.5 Characterization of cultivated *E. coli* isolates

Forty *E. coli* isolates from 30 animals were analysed by using molecular methods. The detailed characterization included virulence genotyping, MLST, phylogenetic typing and testing for resistance towards antimicrobial agents.

3.5.1 Virulence genotyping of *E. coli*

To find out whether *E. coli* and *E. albertii* from bats have a pathogenic potential, 59 virulence genes associated with a variety of pathogenic mechanisms were tested by PCR.
Of the 59 VAGs tested, 36 could be detected in at least one isolate. The number of VAGs per isolate ranged from 5 to 25, irrespective of known potential co-localization of certain genes on plasmids or PAIs. Three isolates (7.7%) harboured a particular high number of VAGs (22-25); nine strains (23.1%) still possessed 11-16 VAGs, whereas the majority of strains (n=27) (69.2%) had 5-10 VAGs, most of which are common in both pathogenic and commensal strains. Identical VAG profiles were found only rarely. The 10 strains, that were initially identified as copy strains by MLST and PFGE analysis harboured the same virulence gene profiles detected in their counterparts. This supports the previous assumption, that they had been sampled twice from the respective animals. To avoid any bias in the interpretation of data, all copy strains were excluded from the following analyses.

Figure 11: Example for an electropherogram of Multiplex PCR I; samples are compared to 2 positive controls (IMT7920, IMT9267)



Almost all strains harboured the adhesion-related genes *fimC* and *mat*, the serum resistance gene *ompA* and the iron acquiring gene *feoB* (94.9%-100%). According to the VAG profile strains were divided into EPEC, ExPEC, ExPEC-like and commensal strains.

Shiga-toxin genes *stx1* and *stx2,* which would be predictive for the presence of STEC, were not present in any of the 40 tested *Escherichia* spp. isolates. Four isolates (IMT25529, IMT25433, IMT26183 and IMT26187) could be assigned to the EPEC pathovar. These isolates harboured the typical EPEC genes *eae*, *escV*, and *bfp,* which are lacking in *E. coli* strains of the common healthy gut flora of mammals. *Eae* and *escV* genes encode for the adhesin intimin and a type III secretion protein, both genes located on the Locus of enterocyte effacement (LEE). *Bfp* encodes for the plasmid-located bundle forming pili. One *E. coli* isolate (IMT26127) was further identified as atypical EPEC (aEPEC) as it was *bfp*- and *stx*-negative but possessed the *eae* and *escV* genes (Kaper, 1996; Trabulsi et al., 2002). Also the *E. albertii* isolate (IMT25440), which was obtained from the lung of a *M. torquata* from PNOK, was tested *eae*+ and *bfp/stx*-. From one *M. torquata* from PNOK with the field no. 12 cultivate both a typical EPEC (IMT25529) from the liver and an aEPEC from the intestine (IMT26127) and lung (copy strain and therefore not listed) were obtained. Three of the six EPEC strains carried the heat stable toxin *astA* (60%), which was the most frequent gene among the VAGs found in the identified EPEC.

The invasion related gene *ibeA* was present in 60% (n=3) of the EPEC strains. *IbeA* has been identified as an important factor contributing to the invasion of *E. coli* in intestinal epithelial as well as in brain microvascular endothelial cells, an initial step in the pathogenesis of neonatal meningitis (Kim, 2006; Che et al., 2011).

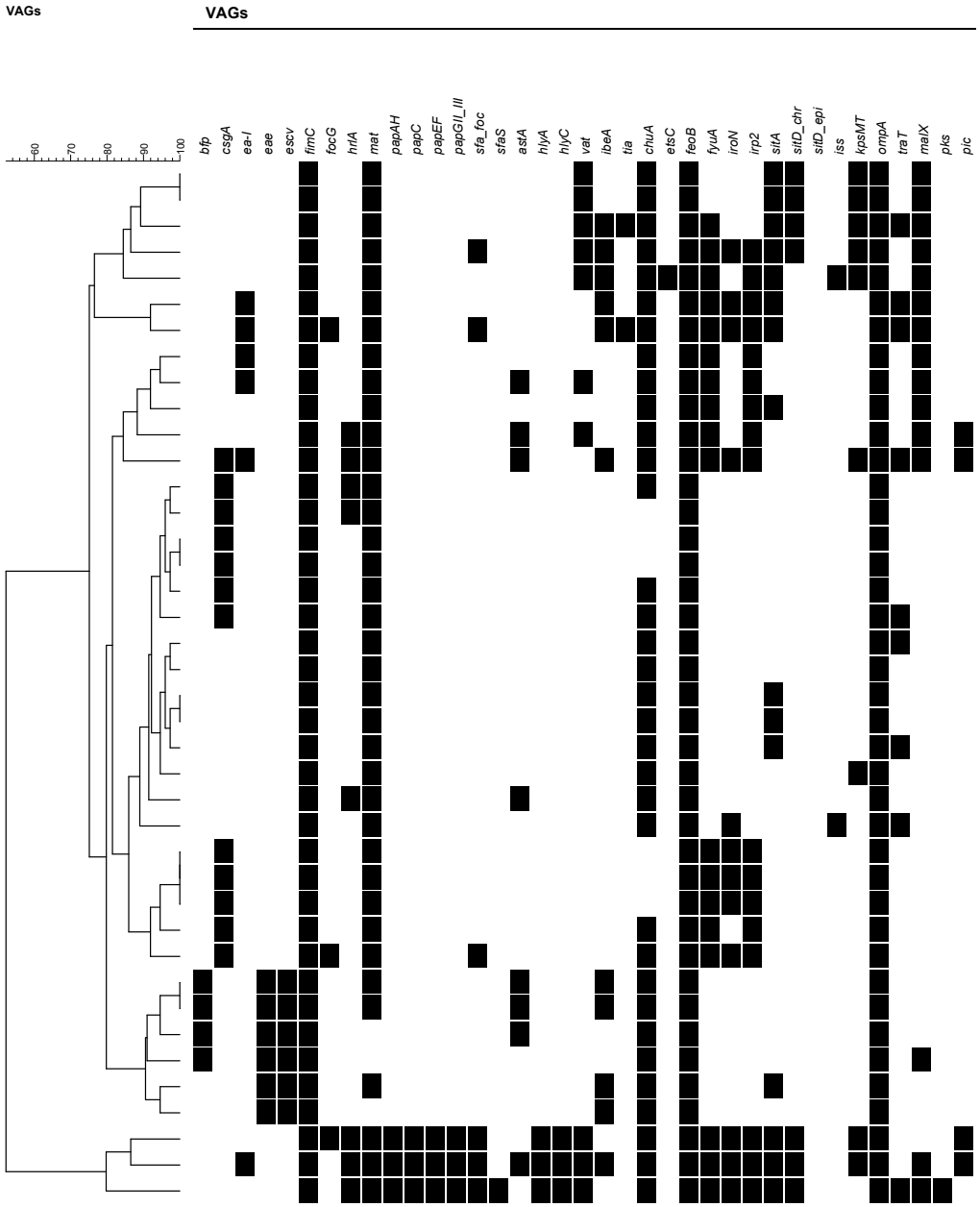
Apart from *chuA* and *feoB,* which were present in all EPEC strains, *sitA* was the only additional iron acquisition gene detectable in one strain. *MalX* only appeared in one EPEC isolate (20.0%).

On average the EPEC strains carried 8.4 VAGs (7-10), which were mainly located chromosomally. *Bfp* and *sitA* were the only representatives of plasmid located genes.

The remaining 34 strains possessed VAGs often associated with ExPEC with a number of VAGs ranging from 5 to 25, including three isolates with more than 20 VAGs. Almost two third (65.9%) of all strains harboured less than ten VAGs, most of which are common in both pathogenic and non-pathogenic strains. Most of the genes were located chromosomally. Plasmid encoded genes were found only to a minor extend.

Excluding *fimC* and *mat*, which were found in all non-EPEC strains tested, *csgA, hrA* and *sfa/foc* were the most frequent adhesion-related genes (32.4%, 23.5%, 17.6% resp.) detected. Three strains possessed the full gene set of P-fimbria, including papAH, papC, papEF and papG II/III. Other adhesion related genes as *sfaS*, *focG* and *yqi* only appeared in a low percentage of 2.9% to 14.7%. The genes *afa/dra, bmaE, gafD, iha, nfaE*, and *tsh* did not occur in any isolate.

Table 13: Virulence gene pattern and phylogenetic groups of 40 *Escherichia* strains (next page); strains of the EcOR group B2 are indicated in red, D in green, B1 in orange, A in dark blue, ABD in middle blue and AxB1 in grey; genes completely absent among strains are not shown in the figure (*afa/dra, bmaE, gafD, iha cds, nfaE, tsh, eitA, eitC, ireA, iucD, iutA, sitD epi, neuC, ompT, cnf1/2, hlyf, sat, stx1, stx2, cvaA, cvi/cva, etsB, gimB, puvA*)



ST	Strain	Pathovar	Field-No	Site	Bat species	Organ(s)
3508	26142	ExPEC-like	15	PNOK	*M. torquata*	Int
2802	26133	ExPEC-like	24	PNOK	*M. torquata*	Int
1597	25887	ExPEC-like	1992	IFO	*M. torquata*	Int
2800	26164	ExPEC	22	PNOK	*M. torquata*	Int
555	27512	ExPEC-like	137	PNOK	*M. torquata*	Int
681	26130	ExPEC-like	24	PNOK	*M. torquata*	Li, Ki
2808	26389	ExPEC-like	2044	IFO	*M. torquata*	Int
2804	27513	ExPEC-like	53	PNOK	*M. torquata*	Int
3507	26186	ExPEC-like	1884	IFO	*H. monstrosus*	Int
2809	26198	ExPEC-like	2008	IFO	*M. torquata*	Int
2806	26181	ExPEC-like	1878	IFO	*R. aegyptiacus*	Int
131	26197	ExPEC-like	1952	IFO	*Ei. helvum*	Int
2810	25441	Commensal	1893	IFO	*M. torquata*	Lu, Ki
937	26401	Commensal	2076	IFO	*M. torquata*	Int
101	26200	Commensal	2033	IFO	*M. torquata*	Int
1408	26203	Commensal	2035	IFO	*M. torquata*	Int
2812	26099	Commensal	1893	IFO	*M. torquata*	Int
657	26201	Commensal	2033	IFO	*M. torquata*	Int
3226	26144	Commensal	14	PNOK	*M. torquata*	Int
3509	26400	Commensal	2075	IFO	*Ep. franqueti*	Int
69	25439	Commensal	24	PNOK	*M. torquata*	Lu, Li, Ki, Int
3228	26109	Commensal	1864	IFO	*M. torquata*	Int
2814	25584	Commensal	1895	IFO	*M. torquata*	Lu, Int
1938	26145	Commensal	1868	IFO	*M. torquata*	Int
2811	26387	Commensal	2042	IFO	*M. torquata*	Int
2805	26107	Commensal	1831	IFO	*R. aegyptiacus*	Int
1146	26195	Commensal	1949	IFO	*Ei. helvum*	Int
1146	26199	Commensal	2031	IFO	*M. torquata*	Int
1146	26399	Commensal	2072	IFO	*M. torquata*	Int
2271	25533	Commensal	1892	IFO	*M. torquata*	Lu
2803	26163	ExPEC-like	29	PNOK	*H. monstrosus*	Int
3225	25529	EPEC	12	PNOK	*M. torquata*	Li
3225	25443	EPEC	1992	IFO	*M. torquata*	Lu, Ki, Int
2807	26183	EPEC	1889	IFO	*Ep. franqueti*	Int
3229	26187	EPEC	1891	IFO	*M. torquata*	Int
722	26127	aEPEC	12	PNOK	*M. torquata*	Int, Lu
3227	25440	*E. albertii* (eae+)	24	PNOK	*M. torquata*	Lu
2801	26177	ExPEC	22	PNOK	*M. torquata*	Int
372	26196	ExPEC	1952	IFO	*Ei. helvum*	Int
127	25442	ExPEC	1893	IFO	*M. torquata*	Lu, Int

The detection rate of toxins was generally low. Most frequent gene encoding for toxin was the vacuolating autotransporter toxin gene *vat* in almost all ExPEC strains and 50% of ExPEC-like strains. The heat stable cytotoxin gene *astA* was present in 20.5% of strains. Hemolysins *hlyA* and *hlyC* were only found in ExPEC-strains and occurred only in strains positive for P-fimbriae. These isolates also had a hemolytic phenotype when cultivated on blood agar. None of the strains harboured the toxin genes *cnf1/2*, *hlyF or sat.*

Seven of the 12 genes encoding for iron acquisition systems were detectable in ExPEC strains. Apart from *chuA* and *feoB*, which were present in 84.6% and 100% resp. of all strains, 38.2% possessed 3-5 additional iron acquisition genes involved in bacterial capture of iron from the host. *FyuA* and *irp2*, both encoding for the siderophore yersiniabactin (co-located on the high pathogenicity island PAI IV on the yersiniabactin-operon) were present in more than 40% of ExPEC strains. Only in one case *fyuA* was detected without *irp2*. Nine of the 18 strains positive for *fyuA/irp2* also harbored the plasmid coded gene *sitA*, encoding for the salmonella iron transport system. In general *sitA* was detected in 38.5% of strains. Almost one third (28.2%) carried the plasmid-linked iron related gene *iroN.* None of the strains possessed *eitA* or *C*, *ireA*, *iucD*, *iutA* or the episomal located *sitD*.

In the group of protectins, the capsule encoding gene *kpsMT II* was present in a percentage of 23.1%, however none of the *kpsMT II* positive strains harbored the K1 capsule gene *neuC*, which is known to be associated with highly virulent and invasive ExPEC strains. Almost one fourth (23.1%) harbored the plasmid for the surface exclusion protein *traT*, whereas the gene involved in increased serum resistance *iss* occurred only in a low percentage of 5.1%.

Other genes such as *malX* were present in 38.5% of strains. *EtsC* and *pic* occurred in a low percentage of 2.6% and 7.7%. In comparison to the high detection rate in EPEC strains, *ibeA* could only be detected in 20.6% of the ExPEC strains. Another invasion associated gene, the toxigenic invasion locus tia was found in a low percentage of 5.1% of all strains.

The plasmid encoded genes *traT* and *iroN* appeared in percentages of 23.1-28.3%, *etsC, iss* and *pks* only in low numbers of one or two strains. *IroN* however can be located on both plasmid and chromosomally, presence of this gene is therefore not necessarily a proof for the existence of plasmid.

Table 14: Distribution of virulence associated genes among *E. coli* from 50 fruit bats

Category/ gene	Positive strains %					Category/ gene	Positive strains %				
	EPEC / aEPEC (n=5)	ExPEC (n=4)	ExPEC-like (n=12)	Commensals (n=18)	Total (n=39)		EPEC / aEPEC (n=5)	ExPEC (n=4)	ExPEC-like (n=12)	Commen-sals (n=18)	Total (n=39)
Adhesins						**Protectins**					
afa/dra	0	0	0	0	0	*iss*	0	0	8.3	5.6	5.1
bmaE	0	0	0	0	0	*kpsMTII*	0	75	41.7	5.6	23.1
bfp	80	0	0	0	10.3	*neuC*	0	0	0	0	0
csgA	0	0	8.3	55.6	28.2	*ompA*	100	100	100	100	100
eae	100	0	0	0	12.8	*traT*	0	25	33.3	22.2	23.1
fimC	100	100	100	100	100	**Toxins**					
focG	0	25	16.7	0	7.7	*EAST-1*	60	25	25	5.6	20.5
gafD	0	0	0	0	0	*cnf1/2*	0	0	0	0	0
hrA	0	75	16.7	16.7	20.5	*hlyA*	0	75	0	0	7.7
iha	0	0	0	0	0	*hlyC*	0	75	0	0	7.7
mat	60	100	100	100	94.9	*hlyF*	0	0	0	0	0
nfaE	0	0	0	0	0	*sat*	0	0	0	0	0
papAH	0	75	0	0	7.7	*stx1,2*	0	0	0	0	0
papC	0	75	0	0	7.7	*vat*	0	100	50	0	25.6
papEF	0	75	0	0	7.7	**Invasins**					
papGII,III	0	75	0	0	7.7	*ibeA*	60	50	41.7	0	25.6
sfa/foc	0	100	16.7	0	15.4	*gimB*	0	0	0	0	0
sfaS	0	25	0	0	2.6	*tia*	0	0	16.7	0	5.1
tsh	0	0	0	0	0	**Others**					
ea-1	0	25	33.3	0	12.8	*cvaC, cvi/cva*	0	0	0	0	0
Iron acquisition						*escv*	100	0	0	0	12.8
chuA	100	100	100	66.7	84.6	*etsB*	0	0	0	0	0
eitA/C	0	0	0	0	0	*etsC*	0	0	8.3	0	2.6
feoB	100	100	100	100	100	*malX*	20	75	91.7	0	38.5
fyuA	0	100	83.3	22.2	46.2	*ompT*	0	0	0	0	0
ireA	0	0	0	0	0	*pic*	0	50	8.3	0	7.7
iroN	0	100	25	22.2	28.2	*pks*	0	25	0	0	2.6
irp2	0	100	75	22.2	43.6	*puvA*	0	0	0	0	0
iucD	0	0	0	0	0						
iutA	0	0	0	0	0						
sitA	20	100	58.3	16.7	38.5						
sitD chr	0	100	25	0	17.9						
sitD epi	0	0	0	0	0						

3.5.2 Multilocus Sequence Typing

MLST was performed for all *Escherichia coli* isolates. Thirty-seven different sequence types (STs) were identified among the 40 non-duplicate isolates.

Fifteen strains could be assigned to previously reported STs, which had already been observed in humans and/ or animals (ST69, ST101, ST127, ST131, ST372, ST555, ST657, ST81, ST722, ST937, ST1146, ST1408, ST1597, ST1938 and ST2271), including strains of known human health relevance (ST69, ST127 and ST131) (table 14).

Twenty-two strains had either new allelic profiles of known alleles or new sequences of one or more alleles. New alleles appeared in all of the seven housekeeping-genes. For those strains new STs were assigned (table 6). Most of the identified strains occurred only singularly, while detection of identical strains was rare. ST1146 was present in three individuals of two different species (*M. torquata, E. helvum*). ST 3225 was found in two individuals of the same species *(M. torquata)* from different regions in north and west of the RC.

To further analyze the genetic relationship between the bat isolates, concatenated gene sequences of the isolated STs were compared to the available *E. coli* STs from the MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli). These analyses were performed by Torsten Semmler, RKI Berlin, Germany.

Whole genome sequence analysis of the ST131 strain revealed that it groups within the cluster B, one of three *E. coli* ST131sublineages. Consistent with the strain´s sensitivity towards fluoroquinolones it shares *gyrA* and *parC* variants as to find in other strains of this clade. Generally and unlike clade C, clade B is not associated with antimicrobial resistances (Petty et al., 2014).

Figure 12: Genetic relationship of 40 *Escherichia* sp. Isolates from African fruit bats. Phylogenetic tree based on concatenated sequences (3423bp) of seven housekeeping genes used for MLST analysis showing the genetic relationship of 40 *Escherichia* sp. isolates from African fruit bats and virulence-gene- based assignment to pathovars. Sequence types (reference STs) frequently associated with ExPEC (ST62, ST95, ST73), EPEC or aEPEC (ST15, ST20, ST28, ST29), and commensals (ST10) (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) are included for comparative purposes. Likewise, members of the "second population" Escherichia clades I-V, of *E. albertii* and *E. fergusonii* are also included (Wirth et al., 2006; Walk et al., 2009; Luo et al., 2011). The maximum likelihood tree was created with PhyML (substitution model HKY85) after alignment of sequences with MAFFT v7.017, both implemented in Geneious. Numbers at nodes represent bootstrap values >70%, expressed as percentages of 1000 replications. Bar: 0.03 substitutions per sequence position.

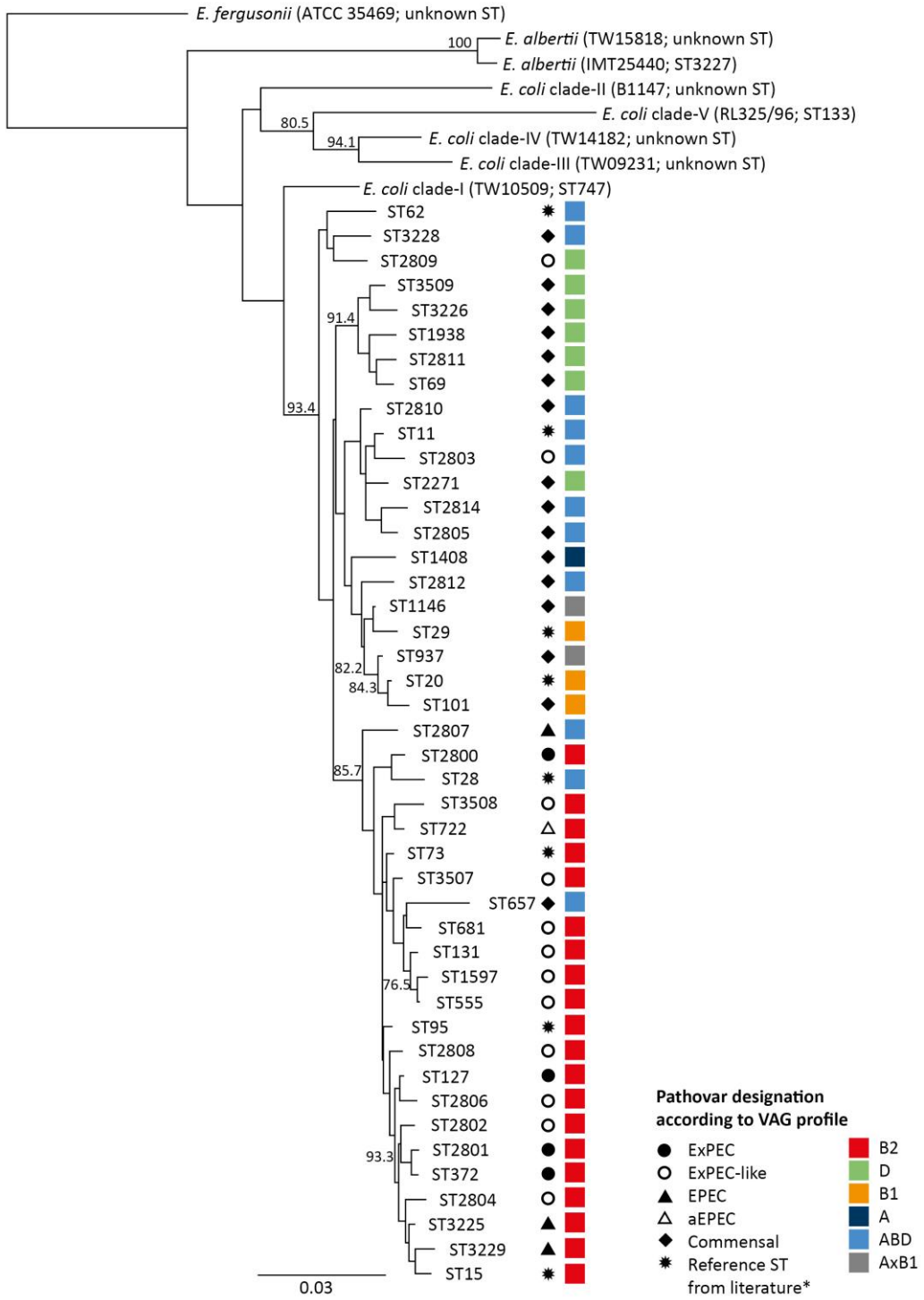
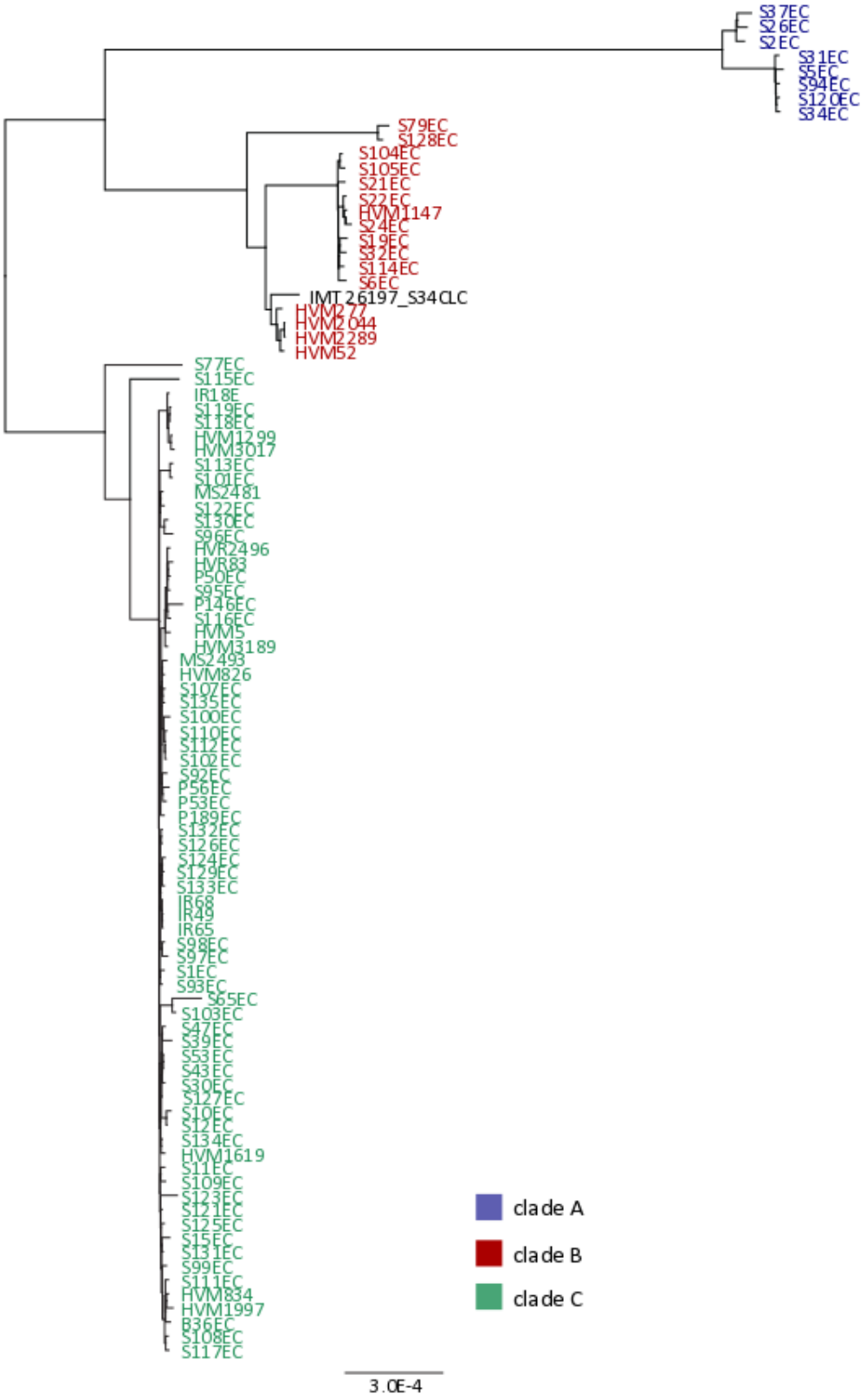


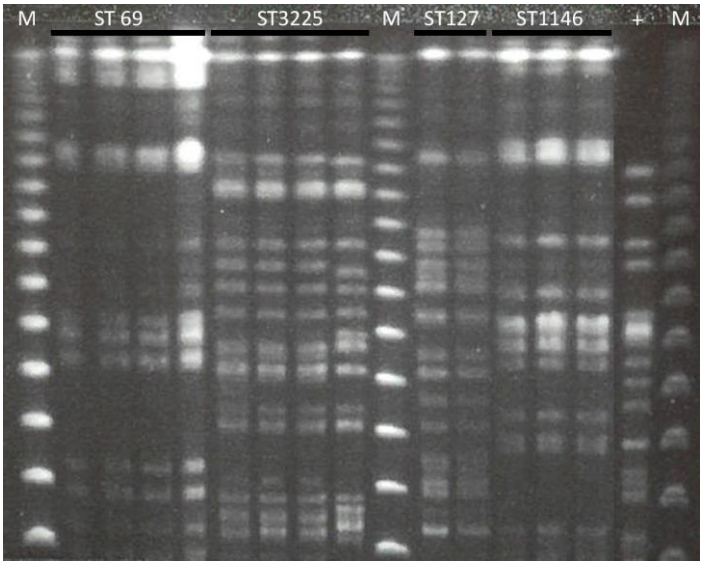
Figure 13: Phylogenetic relationship of ST131 strains on the basis of the maximum common genome (MCG) (Petty et al., 2014) of the ST131 strain (IMT26197) and public available ST131 strains from a comparative study (von Mentzer et al., 2014). Allelic sequences of 3698 orthologous genes where aligned and a maximum likelihood phylogeny was calculated with RAxML8.1. ST131 strains form three clades, clade A, B and C, coloured in blue, red and green respectively. ST131 of this study clusters within clade B.



3.5.3 Pulsed-Field Gel Electrophoresis

PFGE analysis of isolates from different organs within one animal as well as identical isolates deriving from different animals were used to identify copy strains based on the detection of identical STs with identical macrorestriction profiles (Figure 14). As indicated in Table 11, ten out of 50 isolates showed identical PFGE pattern. IMT26100 and IMT25442, both belonging to ST127 were clones too. The three isolates assigned to ST1146 (IMT26194, IMT26199, IMT26399), which came from three different animals of two different species also had a clonal nature. IMT25529, 25443, 25882 and 25886, deriving from two individuals of different regions of the RC could also be identified as clones of the newly assigned ST3225.

Figure 14: CHEF-PFGE-electropherogram of XbaI-restricted genomic DNA selected strains (angle, 1208; voltage 6 V/cm; pulsed-field times 5–12 s for the first 11 h and 20–50 s for the next 11 h; ramping, linear). M: lambda ladder PFG marker; + = positive control

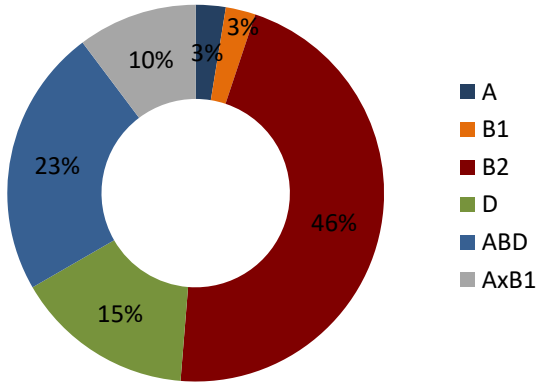


3.5.4 Phylogenetic grouping

The determination of the phylogenetic group membership already gives much information about the characteristics of an unknown *E. coli* strain. Based on the structure analysis of the concatenated sequences of the seven housekeeping genes, amplified during MLST (http://pritch.bsd.uchicago.edu/structure) all strains were assigned to phylogenetic groups A, B1, B2, D and the hybrid groups ABD and AxB1, which evolved from the four main groups.

The 40 isolates were classified into phylogenetic group B2 (46%), ABD (23%), D (15%), AxB1 (10%), B1 (3%) and A (3%) (Figure 15). Isolates belonging to group B2 harboured 13.3 VAGs in average, followed by group ABD (7.6), AxB1 (7.5), D (6.3), A and B1 (5). Genes encoding for p-fimbriae, *ea-I, hlyA*, *hlyC, sit D* (chromosomal) and *vat* were exclusively found in group B2. Other genes such as *ibeA*, *kpsMT II*, *astA*, *malX* and *hrA* were also predominantly found in B2 strains, but also occurred to a lesser extend in strains belonging to group D or ABD. Conversely *csgA* was almost exclusively found in groups other than B2.

Figure 15: Observed frequency of phylogenetic groups among *E.coli* isolated from African bats
(results obtained from structure analysis of the concatenated sequences)



3.5.5 Antimicrobial Resistance

E. coli strains (n=39) were susceptible to most of the tested antimicrobial substances according to clinical breakpoints given in the CLSI (CLSI, 2013, 2015). All isolates were susceptible towards amikacin, gentamicin, amoxicillin/clavulanic acid, ampicillin, cefazolin, chloramphenicol, enrofloxacin, marbofloxacin, sulfamethoxazole/trimethoprime and tetracycline. Thirty-five strains were intermediate resistant to doxycycline; towards the cephalosporines cefovecin and cefalexine 28.2% resp. 20.5% *E. coli* isolates showed intermediate resistance. Also *E. albertii* was susceptible to all tested antimicrobial agents indicating a rather susceptible phenotype, although specific breakpoints are not provided for this species. None of the 40 *Escherichia* spp. strains revealed an ESBL-phenotype.

4 Discussion

In the last decades bats came more and more into the scientific focus as source of several zoonotic diseases (Wibbelt et al., 2007; Jülg et al., 2008; Blehert et al., 2009; Whitaker et al., 2009). However, compared to the multitude on research on viral pathogens, information about bacterial infections in bats is still lacking and often restricted to classical microbiological or serological methods. Especially in tropical regions, which count as the origin of the majority of zoonotic diseases (Jones et al., 2008b) the research effort on bacterial infections is comparatively little.

In this thesis, I gathered tissue samples of free-living African fruit bats of different species and used histopathological, classical microbiological and molecular biological techniques to determine the impact of bacterial infection on the bats' health status, as well as the potential risk bats are posing to other wildlife or human health. *E. coli*, the predominating bacterium found in this study, also represents a bacterium with potential impact on human health and was therefore characterized in more detail.

My findings support the following main conclusions:

1) Free living African fruit bats harbour a broad range of environmental germs with no obvious impact on their health status.
2) Free living African fruit bats showed generally few histological changes. The majority of lesions were found in liver and lung.
3) African fruit bats carry a highly divers and antimicrobial susceptible *E. coli* population, giving evidence that the *E. coli* strains identified display a rather naïve population. STs identified included sequence types (e.g. ST69, ST127, and ST131) and pathotypes (e.g. ExPEC, EPEC and aEPEC), which are known pathogens involved in human and/or animal infections.

4.1 Health status of wild caught bats as determined by histopathology and 16S rDNA PCR

Information about the health status of free-ranging African bats is rare and the few studies available focused on dead or sick animals. For example, Mühldorfer et al. (Mühldorfer et al., 2011a, 2011b) analysed several organs of European bats found dead and reported mainly inflammatory lesions mainly in the lungs (26.9%) and livers (24.8%).
To my knowledge, the study presented here is the first histopathological survey on apparently healthy African bats caught in the wild.
One of the main pathologies observed in this study were hepatic granulomas. In general, hepatic granulomas have numerous infectious and non-infectious causes, including bacteria, viruses, funguses and parasites (Coash et al., 2012). Ziehl-Neelsen or Giemsa staining did not reveal any bacterial structure within the granulomas, suggesting another underlying cause such as parasites. Surprisingly, residuals of a parasite could only be found within a granuloma in one animal, whereas structures of possible parasitic origin were seen in the tissue without any immunological reaction. Especially insectivorous bats are known to harbour a wide variety of endoparasites including nematodes, trematodes, cestodes and many protozoans (Nogueira et al., 2004; Muñoz et al., 2011; Mühldorfer et al., 2011b; Lord et al., 2012). Morphological hints for parasite infection in the intestines were mostly detected in insectivorous bats, however further analyses should be performed to substantiate this notion.

The overall presence of pneumonia was similar to studies from Austria (38.7%) and Czech Republic (34.2%) (Hajkova and Pikula, 2007), however, these studies were conducted on injured or exhausted animals showing respiratory symptoms and are hence not comparable to the results of the present study. Animals of this study were apparently healthy thus the high rate of bats showing histopathological signs of pneumonia is quite surprising. Insectivorous and frugivorous species were equally affected.
The high affection rate might be explained by the morphology of bat lungs, which is (similar to avian lungs) characterized by an enormous relative alveolar surface compared to non-flying mammals and could therefore be seen as a major target organ for numerous viral and bacterial diseases (Maina and King, 1984; Reese et al., 2006). Taking into account that animals of this study were apparently healthy, the high affection rate indicates a high infection pressure in tropical areas. This assumption is supported by the high proportion of spleens showing an

activation of lymphoreticular tissue in 76% (n=90) of all animals. In many cases activation of the spleen was the only detectable lesion, which might be explained by the exceptional immune system of bats (Baker et al., 2013).

Based on the results from histopathology, we selected samples from individual bats showing lesions in histopathology and a subset of samples without any lesions visible for examination by 16S rDNA PCR and subsequent cloning and sequence analyses. These analyses revealed a broad variety of bacteria, among them several of animal or human health-relevance.

The predominating bacteria were members of *Acinetobacter* sp. *Acinetobacter* is a nutritive undemanding and physically very resistant gram-negative bacterium which is on the one hand part of the normal skin microbiota of many humans and animals and broad distributed in the environment; on the other hand several species such as *A. baumannii* or *A. lwoffii* are health-care associated infections comprising bacteraemia, skin and soft tissue infections, wound-, respiratory tract-, urinary tract infections or meningitis. Information about the occurrence of *Acinetobacter* in Africa is rare and mainly restricted to reports of infections with resistant strains in tertiary hospitals (Jeena et al., 2001). *Acinetobacter* couldn't be cultivated in a single sample, which might be due to the lethal injuries of the bacteria during the freezing process.

Another potentially health-relevant bacterium detected was *Bartonella* in one animal. *Bartonellae* are small gram-negative, facultative intracellular bacteria, several of which have been associated with human diseases such as Cat Scratch disease or peliosis hepatis, endocarditis or neuroretinitis. Some of the human strains have also been found in exotic animals, demonstrating the zoonotic character of this pathogen (Inoue et al., 2009). Most *Bartonella* species are transmitted by parasites, but as *Bartonella* could be detected in dog saliva, a transmission through biting is also conceivable (Duncan et al., 2007). *Bartonellae* have worldwide been found in bats or bat flies respectively. Phylogenetic analyses of *Bartonella* strains derived from bats identified several distinct phylogroups indicating the presence of a variety of novel *Bartonella* species in bats (Kosoy et al., 2010; Bai et al., 2011; Lin et al., 2012). More investigation would be needed.

As for the group of Enterobacteriacea*e, E. coli* was present in 17% (n=9) of animals in lung or liver, indicating the presence of extra intestinal pathogenic *E. coli*.

There was no obvious link between bacteria detected by PCR and lesions observed in the histopathology, suggesting lesions to have another causative agent, maybe of parasitic origin that remains to be investigated. Based on the first results described above, the following

analyses focused on the biological and genetic description of *E. coli* in the according bat species.

4.2 Prevalence of *E. coli*

E. coli isolates were recovered from frozen tissue samples from 30 of 50 (60%) African fruit bats.

Comparison to other studies on *E. coli* prevalence in fruit bats is difficult since most studies were performed on captive animals, which doesn't reflect the situation in the wild (Heard et al., 1997; Adesiyun, 1999; Gopee et al., 2000). A higher population density of captive animals combined with closer proximity to humans and thus a different level of exposure to the human microbiome might explain differences in prevalence of *E. coli* in captive compared to free-living animals. Studies on free-living fruit bats only included low sample sizes and are thus also not suitable for generalization (Souza et al., 1999; Adesiyun et al., 2009; Daniel et al., 2013; Lescat et al., 2013).

More studies are available on insectivorous bats with *E. coli* prevalence of up to 28.5%, however differences in prevalence compared to fruit bats might be due to diet and environmental factors (Pinus and Müller, 1980; Gordon and Cowling, 2003; Adesiyun et al., 2009; Apun et al., 2011; Bilung et al., 2014). For example insects, the food source of many insectivorous bat species, have been shown to serve as reservoirs for pathogenic *E. coli* (Khalil et al., 1994; Ahmad et al., 2007).

The influence of the hosts´ diet has also been demonstrated in studies on wild and captive mammals revealing *E. coli* to be less prevalent in carnivorous species compared to herbivorous or omnivorous animals (Gopee et al., 2000; Gordon and Cowling, 2003). Both studies included bats, however the categorization to diet groups in these publications is slightly confusing, as insectivorous were taken as carnivorous and frugivorous as herbivorous species. The dependence on host diet is also supported by results of Pinus and Müller, who demonstrated a higher prevalence of *E. coli* in fruit bats compared to insectivorous bat species (Pinus and Müller, 1980). An explanation might lay in the nutrient availability in the intestinal tracts of different hosts, which obviously differ dependent on the host diet. For example, the carboxylic acid gluconate, which naturally occurs in fruits, appeared to be a major carbon source used by *E. coli*, having an impact on both the initiation and maintenance stages in the host intestinal tract (Chang et al., 2004). *E. coli* strains isolated from bats have also been

demonstrated to exploit a greater range of sugars compared to strains from other mammals (Souza et al., 1999) and might therefore have a special adaptability in different niches and different host species.

Composition of nutrients is not the only variable in the interplay of *E. coli* with the host intestinal tract. The probability of a host to harbour *E. coli* also depends on the frequency that it is exposed to it. Animals living in close proximity to human habitation are more likely to harbour *E. coli* as humans seem to build the biggest reservoir for *E. coli* (Gordon and Cowling, 2003; Lescat et al., 2013). However, for the study presented here, humans are not a likely source of infection of the bats analysed, since all animals were caught in areas with a very low population density (Sierra Leone: 11 inhabitants /km²; Rep. of Congo: 74 inhabitants/km²), which is particularly low in forestry regions like those where animals were trapped. The high detection rate of *E. coli* corroborates the hypothesis of fruit bats not being just transient but permanently populated by *E. coli* as part of their physiological intestinal flora. An explanation for the presence of pathogenic strains in lung or liver might be contamination during the sampling process, post mortem migration or agonal spread of pathogens (Morris et al., 2006). However, samples were taken according to stringent precautions to reduce the possibility of contamination, but of course a residual risk remains.

4.3 Virulence of *E. coli* isolated from fruit bats

VAG typing was performed for the 41 isolates obtained. Of the 59 VAGs tested, 36 could be detected in at least one isolate. The number of VAGs per isolate ranged from 5 to 25. Two third of all strains harboured less than ten VAGs, most of which are common in both pathogenic and non-pathogenic strains.

The present study provides evidence for bats carrying potentially zoonotic *E. coli* pathovars, which are frequently involved in enteropathogenic and extraintestinal diseases in humans. So far, screening for enteropathogenic bacteria in bats mainly focused either on the detection of relevant bacterial species, such as *Campylobacter* spp., *Salmonella*, or, in case of *E. coli*, was often limited to the finding of O157:H7 strains carrying the gene *stx* and thus representing the group of STEC or EHEC (Adesiyun et al., 2009; Apun et al., 2011; Bilung et al., 2014). As STEC represent a relevant cause of severe illness in humans worldwide (Smith et al., 2014) the sample material was also screened for shiga toxin genes. The absence of STEC in the

sample material was in agreement with previous reports. Adesiyun et al., (2009) screened intestinal samples from 377 bats in Trinidad and found none of the 49 *E. coli* isolates recovered from their sample material to be positive for O157:H7 and *stx* genes. Also in two recent studies *stx* genes and O157:H7 *E. coli* could not be detected in bat samples from Malaysia (Apun et al., 2011; Bilung et al., 2014). In addition, direct detection of EHEC-associated genes *stx1*, *stx2*, and *ehxA E. coli* by Real-Time PCR in 412 individual fecal samples from bats in Brazil gave no positive result (Cabal et al., 2015). This study further demonstrates that published negative results might not simply be attributed to the missing of putatively relevant *E. coli* isolates. These studies together with the present results indicate that bats may not be an important reservoir of STEC and EHEC.

Although STEC were not detected among the sample material, other enteropathogenic *E. coli* potentially relevant for human health were identified such as EPEC, aEPEC and also one *eae*-positive *E. albertii*. According to their VAG pattern six isolates were identified as EPEC due to the presence of the *escV* and *eae* genes. Four strains harboured both, the *eae* gene and the EAF-plasmid encoding for *bfp* and were thus categorized as typical EPEC.

Typical EPEC are strongly associated with diarrhoea in children under 1 year, especially in developing countries (Kaper et al., 2004; Okeke, 2009). Humans build the main reservoir of these pathogens whereas reports of typical EPEC from animals are sparse (Nataro and Kaper, 1998; Moura et al., 2009). The main characteristic of EPEC's pathogenicity is the development of typical intestinal attaching/effacing (A/E) lesions, a result of the interaction of several proteins with the intestinal epithelial cells of the host (Kaper et al., 2004), however the expression of A/E lesions depends on a number of physiological and environmental conditions (Rosenshine, 1998).

Information about the pathogenicity of aEPEC is rare and the contribution to human diarrhoea is still controversy discussed. None of the experimentally infected volunteers of a study by Levine et al. (2006) showed clinical symptoms, assuming that aEPEC are less virulent than typical EPEC strains due to the lack of the eaf-plasmid. On the contrary mainly industrialized countries record an increase in diarrheal diseases caused by aEPEC strains, as 90% of all EPEC strains of a study in the UK were lacking the eaf-plasmid (Scotland et al. 1998). Also, even despite the relatively high prevalence of aEPEC in asymptomatic children strains of this pathovar are designated as emerging enteropathogens and are suggested to play an important

role in endemic diarrhea in infants and in outbreaks (Hernandes et al., 2009; Croxen et al., 2013).

Reports of EPEC in wild animals are limited to cervids and monkeys or wild birds (Carvalho et al., 2003; Kobayashi et al., 2009; Bardiau et al., 2010) and so far the occurrence of EPEC in bats has been only once been suggested (Cabal et al., 2015). The data presented here are the first to provide evidence for the presence of typical EPEC and aEPEC in bats. It supports the work of Cabal et al. (2015), who suggested that aEPEC can be carried by bats, as they detected the EPEC-related genes *bfpA* and *eae* by Real-Time PCR in DNA from bats collected in Brazil (Cabal et al., 2015). However, in their study the authors recognized that due to the lack of cultivation of *E. coli,* the detection of multiple genes within pooled samples may not imply that a single bacterium was indeed carrying *bfpA* and *eae* simultaneously (Cabal et al., 2015). Although the *E. coli* strains are physical at hand, this „point-prevalence" study may not provide an answer to whether bats were indeed infected with these pathogenic *E. coli* or were just temporary carriers of these strains.

Data about the presence of VAGs of EPEC other than those located on LEE are rare. AstA, which was present in 43% (n=3) of the EPEC strains, encodes for the enteroaggregative heat stable enterotoxin EAST-1. It is a genetically distinct toxin structurally related to heat-stable enterotoxin I of enterotoxigenic *E. coli* (Savarino et al., 1993) and has been detected in typical and atypical EPEC strains (Müller et al., 2007). The significance of this toxin in EPEC pathogenesis is questionable, as genes encoding for EAST-1 were found in both commensal *E. coli* strains and strains responsible for large outbreaks of diarrhoea in USA and Finland (Hedberg et al., 1997; Nataro and Kaper, 1998). Others suggest even a negative association of astA with diarrhea (Afset et al., 2006).

Invasion gene *ibeA*, which was present in 60 % of EPEC strains, is an important factor that contributes in *E. coli* invasion in intestinal epithelial cells as well as in brain microvascular endothelial cells in the pathogenesis of neonatal meningitis. *IbeA* has also been suggested to play a role in avian pathogenic *E. coli* (Germon et al., 2005). Even though the clinical significance of cell entry in the pathogenesis of disease due to EPEC is not clear, Cieza and colleagues report the contribution of *ibeA* to the interaction of enteroinvasive *Escherichia* with intestinal epithelial cells of murine intestines (Cieza et al., 2015). The direct assessment of the contribution of the mentioned VAGs to virulence would require pathogenicity studies.

Histopathologic analyses of the intestines of the bats carrying EPEC strains revealed no A/E lesions, as they would be typical for EPEC infections. An explanation might be, that sample material used for histopathology than for microbiological analyses derived from different parts of the GIT. Another might be, that the presence of the *eae* gene alone is not sufficient to distinguish the truly pathogenic strains from commensal strains (Knutton et al., 1993; Vieira et al., 2001), often additional VAGs as toxins or invasins are required to lead to disease. A study by Schierack showed that VAGs specific for the EPEC pathotype, such as est-I or II can also be found in *E. coli* from clinically healthy pigs (Schierack et al., 2007).

Another interesting aspect is the finding of an *eae*-positive *E. albertii* strain from the lung of a *M. torquata* bat sampled in the PNOK national park. The clinical significance of *E. albertii* possessing intimin has yet not been fully elucidated. This may partly be due to the difficulty in discriminating *E. albertii* from other *Enterobacteriaceae* spp. by using routine bacterial identification systems based on biochemical properties, as previously discussed by Ooka et al. (2012), who identified 26 eae-positive strains initially determined as EPEC and EHEC as *E. albertii* based on multilocus sequencing analysis. As 13 strains derived from humans that suffered from gastrointestinal infection, *E. albertii* was considered as putative major enteric human pathogen (Ooka et al., 2012).

Apart from EPEC and aEPEC we also identified strains resembling ExPEC or ExPEC-like pathogens. Due to the genetic and clinical complexity of ExPEC and also of different pathovars comprised in this group, such as UPEC, APEC, strains associated with bloodstream infection in humans and mammalian hosts (SePEC) or with meningitis in infants (NMEC), there is no clear definition of ExPEC based on single virulence genes as is the case for several intestinal pathogenic *E. coli* pathovars. Instead, there are multiple and redundant pathogenic mechanisms in this group of opportunistic pathogens. Although probably oversimplifying this complexity, we used a previously suggested molecular definition of ExPEC (Johnson et al., 2003) as an initial basis to identify ExPEC among our bacterial samples. Based on this algorithm, we identified four ExPEC strains from three bats. Three of these isolates also had a haemolytic phenotype when cultivated on blood agar. Further investigation including the genes *ea-I* (Antão et al., 2009), *ibeA* (Homeier et al., 2010), *pks*, *vat* and *sat* (Guyer et al., 2002; Parreira and Gyles, 2003) as well as *hlyA*, *cnf* (Bower et al., 2005) revealed 12 ExPEC-like strains from 11 bats based on the presence of at least five ExPEC-related genes as described in the material and methods section. The pathogenic potential of the isolated strains has not been evaluated in this study, thus a final statement about pathogenicity cannot be

made. In the same way, virulence also cannot be completely ruled out for strains classified as commensals.

The most abundant group of VAGs found were genes encoding for adhesive organelles, such as *fim, pap, sfa* and *foc*, encoding for type 1, P-, S- and F1C- pili, followed by genes involved in iron acquisition. This might result from the anatomical conditions in the bat intestinal tract. As already mentioned, the intestinal tract of bats is three times shorter compared to other mammals of the same size, which can result in a reduced gastrointestinal passage times of up to only 15-20 minutes (Klite, 1965). Ingested bacteria have thus only a narrow time window to successfully adhere to the intestinal epithelial cells and to establish a population in the gastro-intestinal tract before being shed out. Intestinal colonization needs hence additional survival factors such as genes involved in cell adhesion and/or iron metabolism to increase the fitness of the strains within this anatomical and physiological environment. Adhesive organelles are very common in ExPEC, in particular UPEC strains, however, the contribution of each pilus is yet not fully understood (Snyder et al., 2005, 2006).

The reduced gastrointestinal passage time also leads to a very short time frame for *E. coli* to supply itself with essential nutrients such as iron. Its uptake is one of the essential requirements for survival in mammalian tissue and blood, as all iron is bound to proteins of the host, and tissue and blood are depleted of free iron. Additional acquisition systems are thus required to obtain this growth-limiting micronutrient from the environment.

Most of the VAGs identified in this study were localized chromosomally on pathogenic islands, whereas plasmid related genes have only rarely been found. *Bfp* was the only plasmid encoded gene present in EPEC strains, whereas *iroN* and *sitA, traT* and *iss* occurred in a small number of ExPEC or ExPEC-like strains. However, *iroN* can also be located chromosomally, thus the presence of *iroN* is no proof for the presence of a plasmid.

The largely absence of plasmids might be due to the lack of selective pressure in tropical virgin forests. In an intact natural habitat the input of “new” DNA material in terms of the microbial flora is usually a rare event and hence the microbial flora in both, environment and wildlife should stay stable. Souza and colleagues observed that with an increasing human proximity, the frequency of plasmids in *E.coli* strains isolated from animals increases (Souza et al., 1999).

4.4 Typing of isolates and phylogenetic grouping

The results of the MLST analyses support a potentially zoonotic character of the isolated strains, as 15 STs have already been found among *E. coli* isolated from partly severe extraintestinal diseases in humans and/or animals (ST69, ST101, ST127, ST131, ST372, ST555, ST657, ST681, ST722, ST937, ST1146, ST1408, ST1597, ST1938 and ST2271).

As usually the majority of faecal *E. coli* strains in healthy humans and mammals are commensal (Russo and Johnson, 2003), it is remarkable that the examination of this small number of asymptomatic bats (n=50) revealed already at least four *E. coli* isolates known to be involved in partly severe human infections (ST69, ST101, ST127, ST131).

ST69 is globally disseminated and increasingly observed as a multidrug resistant strain causing extra intestinal infections (Tartof et al., 2005; Blanco et al., 2011b). The strain belongs to ECOR group D, containing both, non-pathogenic but also highly virulent variants. The MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) includes to date almost exclusively human UTI strains and only few reports are available about ST69 isolates of animal or environmental origin, mostly commensal strains. The widely absence of VAGs of the present ST69strain suggests a rather non-pathogenic representative, although it was isolated from extra intestinal organs such as lung, liver and kidney. ST101 is a predominantly animal and bird associated ST, which occurs worldwide but is of unknown clinical history. Also this strain is occasionally reported as ESBL-producer or linked to other antimicrobial resistances (Mora et al., 2011). ST127 has so far predominantly been reported as the causing agent for UTIs and other infections in humans, but has several times been observed in domestic and wild animals and shows a high pathogenic potential in animal models (Lavigne et al., 2006; Ewers et al., 2009b; Guenther et al., 2012b; Alghoribi et al., 2014).

The identification of *E. coli* ST131 is of great interest as it is a very successful sequence type, often associated with ESBL-production, which gained worldwide attention due to its pandemic dissemination in humans (Tartof et al., 2005; Nicolas-Chanoine et al., 2008; Blanco et al., 2011b; Mora et al., 2011; Alghoribi et al., 2014) and occasionally in domestic animals and wildlife (Ewers et al., 2012). It is an important cause of severe infections including UTIs, respiratory tract infections, wound infections, gingivitis or enteritis (Johnson et al., 2010), recent studies however relativized the virulence potential by demonstrating an intermediate virulence of ST131 compared to other B2-isolates in different animal infection models (Lavigne et al., 2012; Johnson et al., 2012). ST131 has also been identified in the faecal flora

of healthy people (Leflon-Guibout et al., 2008), all supporting a wide range of virulence diversity within the ST131 .

ST131 is considered one of the main ESBL type CTX-M-15 producing *E. coli*, however it has been shown that ST131 first circulated as a non-ESBL-producing but frequently fluoroquinolone resistant uropathogenic strain for several years (Rogers et al., 2011). The first description of clone ST131 as a producer of ESBL type CTX-M-15 was in 2008 in the UK (Coque et al., 2008; Nicolas-Chanoine et al., 2008). Today reports of non-resistant B2-ST131 strains exist but are rare (Ewers et al., 2010; Johnson et al., 2010). Petty and colleagues (2014) showed that phylogenetically ST131 can be grouped into the three sub lineages A, B and C (Petty et al., 2014). Clade B builds an evolutionary older clade, including non-resistant ST131, in contrast to strains of clade C, which are usually associated with antimicrobial resistance. Most reports of ST131 come from European/industrialized countries, but ESBL producing *E. coli* also pose a rising risk in developing African countries (Clermont et al., 2009; Peirano et al., 2011; Aibinu et al., 2012).

The present ADD test revealed a non-resistant, non-ESBL strain and also whole genome analyses showed that this strain groups within clade B (Figure 13) (Accession number of the strain is deposit at ncbi (LYRV00000000)). Like all clade B strains described previously, also our ST131 strain is characterized by the *fimH22* allele and by *gyrA* and *parC* variants that are consistent with the strain´s susceptibility towards fluoroquinolones (Petty et al., 2014). Generally, this clade is not associated with AMR (unlike clade C). It thus seems that this strain has not been introduced but is rather part of the naïve *E. coli* population in this area, what fits to the aspect, that the isolate derives from an *Eidolon helvum* from a very remote area of the Republic of Congo (IFO), where the human introduction of this strain is unlikely.

Reports of ST131 in animals are sparse but increased during the last few years, especially reports of ESBL-producing strains. Most of these studies focus on domesticated animals or food animals in developed countries (Johnson et al., 2009; Pomba et al., 2009; Mora et al., 2011), where transmission between human and animal is facilitated by close contact. However, ST131 has also been found in companion animals in very remote areas in Kenya, where anthropogenic pressure is low (Albrechtova et al., 2012). Very little data is available about ST131 in wildlife, only few reports come from urban wildlife, more precisely from brown rats and other rodents trapped in human proximity (Guenther et al., 2010a). I here report the first time B2-ST131 in free-living animals in Africa.

In order to identify identical isolates obtained from the samples, PFGE was performed. Although the diversity of STs was generally high, few STs were present in more than one animal. In detail, ST1146 was found in three individuals of two different species (*M. torquata, E. helvum*), both originating from the same region in the northern part of the RC (IFO). PFGE revealed that all ST1146 isolates were clones (Figure 14). The same applied for ST3225, which occurred in two bats of the species *M. torquata* from different regions of the RC (IFO and PNOK) in the North and West of the country, which is distance of several hundred kilometres. Interestingly both isolates were defined as EPEC and revealed identical virulence gene profiles as well as macrorestriction patterns, demonstrating their clonal nature. Both findings clearly indicate that intra- and interspecies transmission of *E. coli* is common in the bat community and strains, pathogenic or not, can easily be transmitted and spread over long distances.

Based on the structure analysis of the concatenated sequences the *E. coli* isolates were assigned to one of the four main phylogenetic groups A, B1, B2, D or the hybrid groups ABD and AxB1 respectively. Nearly half of the isolates (46%, n=18) belonged to phylogenetic group B2, another 15% to group D (n=6), and a high number of isolates were assigned to the hybrid groups ABD (23%, n=9) and AxB1 (10%, n=4). Phylogenetic group B1 and A were represented only by one strain each (3%, n=1). Most of the virulent extraintestinal pathogenic *E. coli* strains are found in group B2 and to a lesser extend to group D. Group B2 strains possess numerous VAGs which enable extraintestinal colonization and maintenance in the host (Clermont et al., 2000). In contrast isolates of group A, B1 and the hybrid groups are usually lacking those genes and are therefore regarded as commensals (Picard et al., 1999; Le Gall et al., 2007). Virulence however is not only determined by the phylogenetic group, as demonstrated in a study by Lavigne et al., who showed that the affiliation to group B2 alone doesn't determine a high virulence (Lavigne et al., 2012). Four of the five EPEC strains in the present study belonged to group B2 and one strain to group D.

In a review by Tenaillon et al. (Tenaillon et al., 2010) phylogenetic group B1 and A seem to be the dominating groups of *E. coli* strains in animals (domesticated and wild), however, few authors report high detection rates for B2-*E.coli* strains in wild animals. Benavides and colleagues found the majority of *E. coli* in wild animals to belong to group D and B2 (Ewers et al., 2009b; Benavides et al., 2012), and also others report of high detection rates of B2-*E. coli* strains in wildlife (Lescat et al., 2013). A comparative study on wild vs. domestic animals from France and several tropical countries showed that *E. coli* isolates of group B2 and D

were more often found in wild animals compared to their domesticated counterparts, however, in general the majority of *E. coli* isolates from wild and domestic animals belonged to group A and B1 (Escobar-Páramo et al., 2006). Smati and colleagues (Smati et al., 2015) however showed no difference in prevalence of B2 and A phylogroups in *E. coli* between domesticated and wild animals.

Another team isolated *E. coli* from asymptomatic zoo animals in Poland and also reports the phylogenetic groups B1 and A to be the dominating groups. Strains of group B2 occurred in only about 10% of all animals tested (Baldy-Chudzik et al., 2008). Strains isolated from zoos, however may not represent the same strains as to find in natural habitats because of a possible contamination of food as well as antimicrobial treatment and re-colonization, which can have a large impact on the intestinal bacterial composition (Clayton et al., 2016).

Information about the distribution of phylogenetic groups of *E. coli* particularly in bats is very limited. In contrast to our results, the majority of *E. coli* isolates from wild-living bats from Australia belonged to phylogenetic group B1 (Gordon and Cowling, 2003). A more recent study on wild animals from Brazil including bats however revealed no difference in the distribution of phylogenetic groups of isolated *E. coli* (Iovine et al., 2015). Animals of the present study seem to harbour a remarkably high percentage of B2- *E. coli* isolates.

Besides their phenotypic and genotypic differences, the phylogenetic groups appear to have different ecological niches and life history characteristics; as claimed for the presence of *E. coli* itself host diet, body mass and gut morphology also seem to determine the prevalence of the different phylogenetic groups (Gordon and Cowling, 2003; Escobar-Páramo et al., 2006; Baldy-Chudzik et al., 2008). Gordon and Cowling claimed that carnivorous species are less likely to harbour B2 strains, followed by herbivorous and omnivorous species. These results support the data of the present study since the phylogenetic group B2 and D as the dominating groups in free-ranging fruit eating bats.

As for body mass and gut morphology bats seem to play a special role. Gordon and Cowling suggest that B2-*E. coli* strains seem to favour complex hind gut morphologies, however such a modification is lacking in bats. Quite the contrary is the case: bats have even a shortened intestinal tract compared to other mammals of the same size (Klite, 1965), which in turns indicates, that B2 strains should be less likely to be isolated from bats intestinal tracts.

Isolates belonging to phylotype B2 harboured the highest number of VAGs (median 10.5), followed by groups ABD (8.0), AxB1 (8.0), D (6.5), A and B1 (5.0). Phylogenetic grouping

and the virulence gene patterns of the *E. coli* strains isolated give evidence that bats could constitute a further reservoir of potentially pathogenic *E. coli.*

4.5 Antimicrobial resistances

None of the isolated strains including those, which are typically associated with ESBL-production (ST69 and ST131) (Coque et al., 2008; Nicolas-Chanoine et al., 2008; Clermont et al., 2008; Blanco et al., 2011a) showed resistance towards the tested antimicrobial agents except for few isolates which showed intermediate resistance to cefovecin and cefalexine.
The result is not surprising as mainly human proximity and human population density are suggested to be responsible for presence and increase of antimicrobial resistances in animals (Gilliver et al., 1999; Österblad et al., 2001; Gordon and Cowling, 2003; Skurnik et al., 2006). For example the majority of the bacterial isolates from mice and voles captured in rural England with a relatively high human population density showed resistance to β-lactam antibiotics, whereas the faecal enterobacteria of wild elk, deer and voles in Finland had almost no resistance (Österblad et al., 2001).
Animals of this study all derived from countries with very low human population density. The Republic of Congo has a population density of 11 inhabitants per square kilometre, and density is even lower in forestry areas remote from human activity and where access is only possible by boat or a several kilometre walk. Exposure to resistant bacteria of human origin is thus negligible in these regions. However the situation of antimicrobial resistances in wildlife is very complex and cannot just be explained by a “No-man, no resistance” theory. Several studies focus on antimicrobial resistances in wildlife (Literak et al., 2010; Guenther et al., 2010b, 2011) mainly targeting on wild animals originating in European countries. Information on the prevalence of antimicrobial resistances in tropical wildlife (especially on bats) is very limited. Multi drug resistant bacteria have been found in several wild animals despite the absence of antibiotic exposure in areas of remoteness comparable to the sample sites of this study (Bartoloni et al., 2004; Sjölund et al., 2008; Wheeler et al., 2012; Guenther et al., 2012a) such as Mongolia or the Arctic. In these cases presence of resistant strains might be explained by the migratory routes of the tested wild birds, which can easily acquire genetic material on their journey and serve as vectors for resistant bacteria. In contrast Albrechtova and colleagues showed the widely absence of antimicrobial resistant *E. coli* inside a rain

forest in Côte d'Ivoire despite the presence of villages close to the border (Albrechtova et al., 2014).

Only few studies investigated antimicrobial resistances in *E. coli* isolated from bats (Souza et al., 1999; Adesiyun et al., 2009). In contrast to our results they found a high frequency of antimicrobial resistances towards ampicillin, streptomycin and erythromycin and lower resistance rates towards neomycin and sulfa/trimethoprim. However to date no ESBL have been found in *E. coli* isolated from bats.

The complete absence of resistance to any of the antimicrobial agents tested indicates that bats of our study did probably not acquire these strains from humans or domesticated animals, but that known human strains are more frequently to be found in wild animals as originally assumed as part of the naïve *E. coli* population.

4.6 General Discussion

Human behaviour and the intrusion in pristine ecosystems have obviously a huge impact on the emergence of new pathogens (Daszak et al., 2001). Logging, the land use of cleared areas, habitat fragmentation and poaching intensifies the contact between wildlife and humans resulting in a higher likelihood for exchange of microorganisms between humans and wildlife. Global trade and travel including the increasing ecotourism industry to remote areas support the worldwide spread of bacteria (and other microorganisms) including *E. coli*.

In this study a high diversity of *E. coli* STs were identified in fruit bats from remote areas of the Republic of Congo, among those several to date unknown STs, but also several known to cause infection in humans and animals. However, numbers of virulence genes were generally low and none of the strains showed resistances to the antimicrobials tested. In addition, the isolated ST131 belonged to a phylogenetically older clade of ST131, which is usually not linked to antimicrobial resistance. The lack of antimicrobial resistance but also the low number of VAGs suggests that the STs identified are not a result of human introduction.

Since several years bats are a known reservoir for a multitude of pathogens relevant for human and animal health. Terrestrial animals are generally more often exposed to *E. coli* since infection usually occurs through an oral-faecal transmission pathway. Arboreal species with rare ground contact, such as bats, seem thus less likely to get in contact with *E. coli* of the surrounding fauna. Occasionally it may be possible that fruit bats land on the ground to

collect fallen fruits, and there is even one report of Indian fruit bats eating soil (Mahandran et al., 2016), however this behaviour could only be observed once. “Infection” via intermediate hosts seems thus to be more likely, since bats often share their habitat and fruit sources with other wild animals such as monkeys or birds. For instance the involvement of migratory birds has been suggested as a source of infection for wildlife in remote areas, where resistant strains of *E. coli* were found despite the absence of antibiotic exposure (Wheeler et al., 2012; Guenther et al., 2012a).

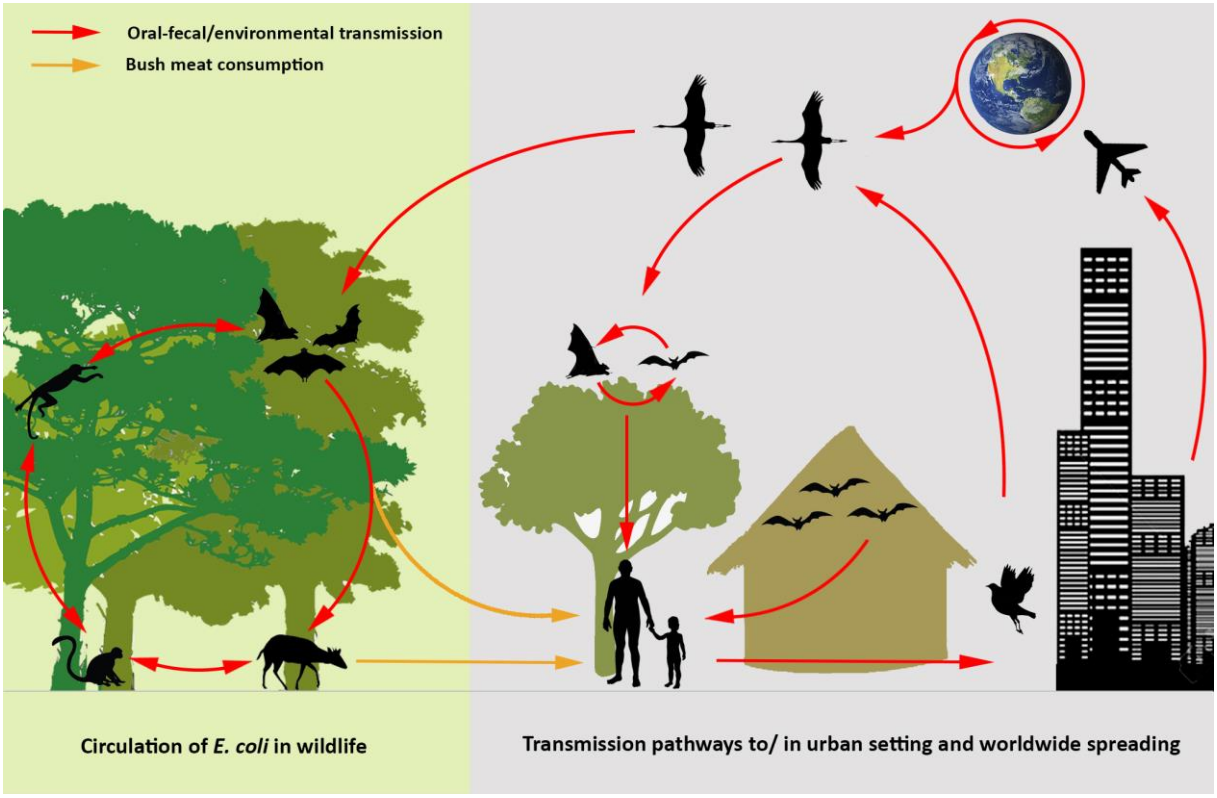
Great population densities as well as the crowded roosting behaviour of some bat species increase the chances of intra- or interspecies transmission of bacteria. However, if transmission within a population would be a frequent event, detection of the same strain in different individuals would probably occur more often than observed in the present study. The high prevalence of *E. coli* in bats indicates a commensal character of this bacterium, making epidemiological considerations less important.

Still, transmission from bats to humans could occur via several pathways. For instance fruit eating bat species can seasonally be found in banana or mango plantations where they could contaminate fruits (half eaten or masticated fruits), which might be eaten by humans or terrestrial animals. Fruits or their products have already been demonstrated as route of transmission as raw date palm sap has e.g. been identified as the most common pathway of Nipah virus transmission from Pteropus bats to humans (Luby and Gurley, 2012).

One species, *E. helvum,* a migratory bat, is frequently found in urban settings during resting periods on their annual migration (Richter and Cumming, 2008), where faecal contamination of the environment including food markets occurs readily and can easily affect humans and other animals. Acquisition of resistant bacteria from humans is possible, although a transmission of microorganisms from humans to these animals seems unlikely due to the aforementioned reasons.

In contrast to fruit bats, microchiropteran bats often roost in big colonies of several hundred animals under the roof of human dwellings – transmission might occur through faecal contamination of personal belongings or food. The complexity of transmission pathways is illustrated in Fig. 16.

Figure 16: Potential transmission pathways of microorganisms within wildlife, between animals and humans and the worldwide spread



As mentioned above bush meat consumption is probably one of the most important drivers for transmission of foodborne pathogens such as *E. coli* as bats provide a considerable protein source for people in many African countries, where they are systematically hunted and sold (Hennessey and Rogers, 2008; Mickleburgh et al., 2009; Kamins et al., 2011; Vora et al., 2014). For example, in Ghana the consumption of the straw-coloured fruit bat *E. helvum* is estimated at 128.000 animals per year with upward tendency (Kamins et al., 2011; Vora et al., 2014). With the decline of larger wild animals in proximity to human settlement and bigger cities, the rate of hunting for small mammals is increasing; therefore special attention should be given to microorganisms in such mammals (Fa et al., 2013).

The discovery of new zoonoses is often related to better diagnostic tools becoming available (Halpin et al., 2007; Jones et al., 2008a). Ideally pathogens are described before entering the human population (Calvignac-Spencer et al., 2012) and therefore the identification of pathogens of potential impact for human health in animal reservoir will enhance our capacity for the timely detection of zoonoses and therefore contribute to disease control.

5 Outlook

This study presented here gives a first glimpse into the diversity of *E. coli* to be expected in the large taxon of bats. Still, these first results indicate that bats should be considered as another source for *E. coli* and other bacteria of potential relevance for human and animal health.

In order to pinpoint transmission events, future studies, based on high throughput methods, should focus on settings with well-defined human - fruit bat contact frequencies, where systematically human and bat biologic materials can be collected. Such settings could be bushmeat markets, bat hunter communities but also human populations living in proximity to large bat colonies as found in many African cities.

Laboratory analyses should include modern tools such as whole genome sequence analysis to reliably pinpoint transmission events, no matter if the entire organisms (e.g. *E. coli*) are targeted or only DNA fragments such as plasmids, which can cross barriers as well.

However when studying emerging infectious diseases various technical approaches should be applied, ranging e.g. from epidemiological, to histopathological and molecular investigation.

Such studies should always be based on the principal of the One-Health concept and should be performed in close cooperation with local partners and governments.

Appendix

A.1 Primers used for virulence gene identification

Annex table 1: Primers used for virulence gene identification; MP = Multiplex PCR

Abbreviation/ gene	Description	Primer no.	Product (bp)	Sequence (5'-3', sense (s), antisense (as))	Reference
MP I					
afa/draBC	afimbrial, Dr specific adhesin	1604/1605	810 bp	s: TAAGGAAGTGAAGGAGCGTG as: CCAGTAACTGTCCGTGACA	(Ewers et al., 2007)
fimC	Typ 1 fimbria	33/34	477 bp	s: GGGTAGAAAATGCCGATGGTG as: CGTCATTTTGGGGGTAAGTGC	(Ewers et al., 2007)
hrA/hek	heat resistant hemagglutinin	1546/1547	537 bp	s: GTAACTCACACTGCTGTCACCT as: TCACTTGCAGACCAGCGTTTC	(Ewers et al., 2007)
sfa/foc	s-fimbriae adhesin/ F1 fimbriae	1566/1567	1242 bp	s: CGGAGAACTGGGTGCATCTTA as: GTCCTGACTCATCTGAAACTGCA	(Ewers et al., 2007)
hlyA	hemolysin A	21/22	352 bp	s: GTCCATTGCCGATAAGTTT as: AAGTAATTTTTGCCGTGTTTT	(Ewers et al., 2004a)
pic	serin protease autotransporter	1071/1072	412 bp	s: ACTGGATCTTAAGGCTCAGG as: TGGAATATCAGGGTGCCACT	(Ewers et al., 2007)
kpsMTII	group II capsule antigen	1544/2084	270 bp	s: CATCCAGACGATAAGCATGAGCA as: GCGCATTTGCTGATACTGTTG	(Johnson and Stell, 2000)
neuC	K1 capsular polysaccharide	1904/1905	676 bp	s: GGTGGTACATTCCGGGATGTC as: AGGTGAAAAGCCTGGTAGTGTG	(Zapata et al., 1992)
RPai (maIX)	pathogenicity associated island marker CFT 073	1610/1611	922 bp	s: GGACATCCTGTTACAGCGCGCA as: TCGCCACCAATCACAGCCGAAC	(Johnson and Stell, 2000)
MP II					
chuA	*E. coli* haem utilization	445/446	278 bp	s: GACGAACCAACGGTCAGGAT as: TGCCGCCAGTACCAAAGACA	(Clermont et al., 2000)
gimB	genetic island associated with meningitis	1261/1262	736 bp	s: TCCAGATTGAGCATATCCC as: CCTGTAACATGTTGGCTTCA	(Ewers et al., 2007)

ibeA	invasion of brain endothelium	1056/1764	342 bp	s: TGGAACCCGCTCGTAATATAC as: CTGCCTGTTCAAGCATTGCA	(Ewers et al., 2007)
iroN	iron receptor	1608/1609	847 bp	s: ATCCTCTGGTCGCTAACTG as: CTGCACTGGAAGAACTGTTCT	(Ewers et al., 2007)
ompA	outer membrane protein	904/905	919 bp	s: AGCTATCGCGATTGCAGTG as: GGTGTTGCCAGTAACCGG	(Ewers et al., 2007)
traT	transfer protein	902/903	430 bp	s: GTGGTGCGATGAGCACAG as: TAGTTCACATCTTCCACCATCG	(Ewers et al., 2007)
sitD (chrom.)	salmonella iron transport system gene	1765/1766	554 bp	s: ACTCCCATACACAGGATCTG as: CTGTCTGTGTCCGGAATGA	(Ewers et al., 2007)
sitD (episomal)	salmonella iron transport system gene	1762/1763	1052 bp	s: TTGAGAACGACAGCGACTTC as: CTATCGAGCAGGTGAGGA	(Ewers et al., 2007)
MP III					
astA (East-1)	heat stable cytotoxin associated with enteroaggregative *E. coli*	7/8 u. 762/763	116 bp	s: TGCCATCAACACAGTATATCC as: TAGGATCCTCAGGTCGCGAGTGACGGC	(Yamamoto and Echeverria, 1996)
colV (cvi/cva)	structural genes of colicin V operon	1828/1829	598 bp	s: TCCAAGCGGACCCCTTATAG as: CGCAGCATAGTTCCATGCT	(Ewers et al., 2007)
irp2	iron repressible protein	15/1066	413 bp	s: AAGGATTCGCTGTTACCGGAC as: AACTCCTGATACAGGTGGC	(Schubert et al., 1998)
iss	increased serum survival	1847/1848	309 bp	s: ATCACATAGGATTCTGCCG as: CAGCGGAGTATAGATGCCA	(Ewers et al., 2007)
iucD	yersinia bactin	23/24	714 bp	s: ACAAAAAGTTCTATCGCTTCC as: CCTGATCCAGATGATGCTC	(Janssen et al., 2001)
papC	pilus associated with pyelonephritis	31/32	501 bp	s: TGATATCACGCAGTCAGTAGC as: CCGGCCATATTCACATAAC	(Janssen et al., 2001)
tsh	temperature sensitive hemagglutinin	1028/1029	824 bp	s: ACTATTCTCTGCAGGAAGTC as: CTTCCGATGTTCTGAACGT	(Ewers et al., 2007)
vat	vacuolating autotransporter toxin	1069/1070	981 bp	s: TCCTGGGACATAATGGTCAG as: GTGTCAGAACGGAATTGTC	(Ewers et al., 2004a)
MP IV					
cnf1/2	cytotoxic necrotizing factor	11/12	446 bp	s: TCGTTATAAAATCAAACAGTG as: CTTTACAATATTGACATGCTG	(Ewers et al., 2004a)
csgA	"curlin subunit gene A" (Curli fimbria)	1865/1866	250 bp	s: ACTCTGACTTGACTATTACC as: AGATGCAGTCTGGTCAAC	(Maurer et al., 1998)
feoB	ferrous iron transport	1664/1665	1095 bp	s: TGAGGACTCCGGCTATATGG as: ATAACTGCCAGAATGCACACC	(Römer, 2009)

fyuA	ferric yersinia uptake (yersiniabactin receptor	13/14	775 bp	s: GCGACGGGAAGCGATGACTTA as: CGCAGTAGGCACGATGTTGTA	(Schubert et al., 1998)
ireA	iron responsive element	1867/1868	384 bp	s: ATTGCCGTGATGTGTTCTGC as: CACGGATCACTTCAATGCGT	(Ewers et al., 2007)
mat	meningitis associated and temperature regulated fimbriae	2086/2087	899 bp	s: TATACGCTGGACTGAGTCGTG as: CAGGTAGCGTCGAACTGTA	(Ewers et al., 2007)
sat	secretet autotransporter toxin	840/841	667 bp	s: TGCTGGCTCTGGAGGAAC as: TTGAACATTCAGAGTACCGGG	(Ewers et al., 2007)
tia	toxigenic invasion locus	1941/1942	512 bp	s: AGCGCTTCCGTCAGGACTT as: ACCAGCATCCAGATAGCGAT	(Ewers et al., 2007)
MP V					
bfp	bundle forming pili	3087/3088	910 bp	s: GACACCTCATTGCTGAAGTCG as: CCAGAACACCTCCGTTATGC	(Müller et al., 2007)
escv	escherichia secretion complex	3085/3086	544 bp	s: ATTCTGGCTCTCTTCTTCTTTATGGCTG as: CGTCCCCTTTTACAAACTTCATCGC	(Müller et al., 2006)
stx1	shigatoxin 1	3089/3090	244 bp	s: CGATGTTACGGTTTGTTACTGTGACAGC as: AATGCCACGCTTCCCAGAATTG	(Müller et al., 2006)
stx2	shigatoxin 2	3091/3092	324 bp	s: GTTTTGACCATCTTCGTCTGATTATTGAG as: AGCGTAAGGCTTCTGCTGTGAC	(Müller et al., 2007)
MP VI b					
focG	fimbriae of serotype 1C	3174/3175	360 bp	s: CAGCACAGGCAGTGGATACGA as: GAATGTCGCCTGCCCATTGCT	(Johnson and Stell, 2000)
gafD	N-acetyl-D-Glucosaminspezifisches fimbrial lectin	3178/3179	952 bp	s: TGTTGGACCGTCTCAGGGCTC as: CTCCCGGAACTCGCTGTTACT	(Johnson and Stell, 2000)
iutA	iron uptake transport	3182/3183	300 bp	s: GGCTGGACATCATGGGAACTGG as: CGTCGGGAACGGGTAGAATCG	(Johnson and Stell, 2000)
nfaE	nonfimbrial adhesin	3180/3181	559 bp	s: GCTTACTGATTCTGGGATGGA as: CGGTGGCCGAGTCATATGCCA	(Johnson and Stell, 2000)
papAH	pilus associated with pyelonephritis	3157/3158	721 bp	s: ATGGCAGTGGTGTCTTTTGGTG as: CGTCCCACCATACGTGCTCTTC	(Johnson and Stell, 2000)
MP VI c					
bmaE	blood group M-specific adhesin	3176/3177	505 bp	s: ATGGCGCTAACTTGCCATGCTG as: AGGGGGACATATAGCCCCCTTC	(Johnson and Stell, 2000)
hlyC	hemolysin C	4199/4200	556 bp	s: AGGTTCTTGGGCATGTATCCT as: TTGCTTTGCAGACTGCAGTGT	(Bingen et al., 1998)

papEF	pilus associated with pyelonephritis	3159/3160	336 bp	s: GCAACAGCAACGCTGGTTGCATCAT as: AGAGAGAGCCACTCTTATACGGACA	(Yamamoto et al., 1995)
papGII, III	pilus associated with pyelonephritis	3161/3162	1057 bp	s: CTGTAATTACGGAAGTGATTTCTG as: ACTATCCGGCTCCGGATAAACCAT	(Marklund et al., 1992)
sfaS	s-fimbriae adhesin	3172/3173	242 bp	s: GTGGATACGACGATTACTGTG as: CCGCCAGCATTCCCTGTATTC	(Johnson and Stell, 2000)
MP VII					
hlyF	hemolysin F	3818/3819	250 bp	s: ATGGATCCTCGTCTTGATG as: GCTCATTGCCAGTATGTCA	(Nowak et al., 2016)
ompT	outer membrane protein	3820/3821	610 bp	s: TTGCTACTGCACTCTCAGC as: CGACAGATACTCTGGGTAACA	(Nowak et al., 2016)
pks	polycetide synthase	2540/2541	300 bp	s: CGCTTCATCAACACGCTTTA as: CCATCGCCTATCACCTCAAC	H. Karch
MP VIII					
iha	iron regulated gene-homologe adhesin	1943/1944	2088 bp	s: ATGCGAATAACCATTTTGGCTTCC as: TCAGAACTGATAGTTCAGCGACA	(Nowak et al., 2016)
puvA	previously unidentified virulence gene	1602/1603	521 bp	s: CACACTTGTATAGCAACCCGT as: TGCACTGTCAGATACAGGTG	(Nowak et al., 2016)
ea/ I	novel adhesin	2512/2559	761 bp	s: ATGCAATGGCAGTACCCTTC as: ATAAACACAATATGGCGCTCG	(Antão et al., 2009)
MP IX					
cvaC	colV-plasmid-structure protein	4195/4196	679 bp	s: CACACACAAACGGGAGCTGTT as: CTTCCCGCAGCATAGTTCCAT	(Johnson and Stell, 2000)
eitA	*E. coli* iron transport	4185/4186	450 bp	s: ACGCCGGGTTAATAGTTGGGAGATAG as: ATCGATAGCGTCAGCCCGGAAGTTAG	(Johnson et al., 2006)
etsA	*E. coli* transport system	4189/4190	284 bp	s: CAACTGGGCGGGAACGAAATCAGGA as: TCAGTTCCGCGCTGGCAACAACCTAC	(Johnson et al., 2006)
etsB	*E. coli* iron transport	4191/4192	380 bp	s: CAGCAGCGCTTCGGACAAAATCTCCT as: TTCCCCACCACTCTCCGTTCTCAAAC	(Johnson et al., 2006)
eitB	*E. coli* transport system	4187/4188	537 bp	s: TGATGCCCCGCCAAACTCAAGA as: ATGCGCCGGCCTGACATAAGTGCTAA	(Johnson et al., 2006)
sitA	*salmonella* iron transport system gene	4193/4194	608 bp	s: AGGGGGCACAACTGATTCTCG as: TACCGGGCCGTTTTCTGTGC	(Rodriguez-Siek et al., 2005)

A.2 Allelic profiles

Annex table 2 Allelic profiles of new assigned sequence types (STs); new sequence numbers are coloured in red

ST	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*
2800	13	363	49	14	17	199	34
2801	88	43	9	334	17	44	96
2802	76	103	247	335	30	10	228
2803	12	406	298	30	15	2	2
2804	13	43	9	37	118	252	85
2805	83	260	299	12	1	1	2
2806	13	14	19	22	30	11	229
2807	76	407	10	336	30	14	230
2808	13	24	19	36	262	14	25
2809	17	37	13	96	26	146	91
2810	80	23	42	12	1	2	2
2811	164	22	160	6	137	16	19
2812	6	260	225	291	80	1	138
2813	13	24	10	14	30	14	92
2814	87	276	53	24	55	178	2
3225	13	43	19	15	17	14	238
3226	203	434	39	212	240	232	239
3227	316	200	317	98	256	149	103
3228	251	88	318	29	279	127	11
3229	76	435	10	360	30	14	25
3507	327	52	19	37	16	289	25
3508	15	460	190	13	296	30	96
3509	35	22	52	27	5	2	218

A.3 Staining protocols for histopathology

A.3.1. Deparaffinisation and Hydration

- Xylol – 2 x 10min
- Ethanol 99% – 2 x 3min
- Ethanol 96% – 3min
- Ethanol 80% – 3min
- Celloidin – Rinse
- Ethanol 70% – 3min
- Aqua dest. – 3min

A.3.2. Dehydration and Embedding

- Aqua dest. – rinse
- Ethanol 70% - rinse
- Ethanol 80% - rinse
- Ethanol 96% - rinse
- Ethanol 99% - 2min
- Xylol – 2 x 5min
- Embedding in Roti-Histol®

A.3.3. Hematoxylin-Eosin staining

- Deparaffinisation and rehydration (see A.3.1.)
- Hämalaun after Mayer – 2min
- Tap water (lukewarm) – 5min
- Aqua dest. – Rinse
- Aqueous Eosin 1% - 3min
- Dehydration and embedding (see A.3.2.)

A.3.4. Giemsa staining

- Deparaffinisation and hydration (see A.3.1.)
- Giemsa solution (10ml Giemsa + 90ml Aqua dest. pH 7.2 – 1h
- 1.0 % acetic acid – Rinse until a slight reddish glow
- Ethanol 96% – blue staining
- Ethanol 99% - rinse
- Xylol – 5 min
- Embedding in Roti Histol®

A.3.6. Prussian Blue staining (proof of iron)

- Deparaffinisation and hydration to distilled water (see A.3.1.)
- Potassium ferrocyanide 2% (2.29g/100ml) + hydrochloric acid 25% 1:1 – 20min
- Aqua dest – rinse well
- Nuclear-fast Red – 5min

- Aqua dest – rinse 3x
- Dehydration and embedding (see A.3.2.)

A.3.5. Ziehl-Neelsen (modified according to Henriksen)

- Deparaffinisation and rehydration (see A.3.1.)
- Carbo-fuchsine 40°C – 60min
- Hydrochloric acid alcohol (100ml ethanol 70%+ 0.25ml hydrochloric acid) – till no colored clouds appear anymore
- Tap water – rinse well
- Methylen blue (1%) – 5min
- Tap water – rinse well
- Dehydration and embedding (see A.3.2.)

A.3.7. Von Kossa stain (calcium staining)

- Deparaffinisation and hydration to distilled water (see A.3.1.)
- 5% Silver solution, place in bright sunlight, or in front of a 60-watt lamp, place foil (or mirror)
- behind the jar to reflect the light. Leave for 1 hour or until calcium turns black.
- Rinse in distilled water, 3 changes.
- 5% Hypo, 5 minutes
- Wash in tap water, rinse in distilled
- Nuclear-fast Red, 5 minutes
- Wash in water
- Dehydration, Embedding (see A.3.2.)

A.3.8. Warthin – Starry

- Prepare:

  A: 4.1g sodium acetate/250ml aqua dest.

  B: 11.8ml glacial acetic acid/1000ml aqua dest

  Stock solution (= Buffer): 1.5ml A + 18.5ml B to 500ml aqua dest

  - Preheat cuvette with 1% $AgNO_3$ in buffer (100ml), to 60°C,

- Preheat 2 cuvettes with tap water to 60°C
- Preheat 1 empty cuvette to 60°C (wrapped in aluminium foil

Developing solution: 2%$AgNO_3$ (0.3g $AgNO_3$in 15ml buffer), 6g gelatine/ 90 ml buffer, 0.3g hydroquinone/ 8ml buffer

- Deparaffinisation and hydration to distilled water (see 9.1.1)
- 1% $AgNO_3$ solution (60°C) – 1h
- Developer solution: 90ml gelatine solution (6g) + 15ml 2% $AgNO_3$ + 8ml hydroquinone in a preheated cuvette
- Dip slides and move up to a golden brown colour
- Warm water - 2x
- Buffer – rinse 3x
- 1% sodium thiosulfate – 2minutes
- Dehydration, embedding (see A.3.2.)

A.3.9. Periodic –Acid-Schiff Reaction

- Deparaffinisation and hydration to distilled water (see A.3.1.)
- 0.5% Perjodsäure – 10min
- Aqua dest. – 10 min
- Schiff's reactive – 15min
- Aqua dest. – rinse some seconds
- Tap water (running) – 10min
- Haemalaun – 30sec
- Tap water – 10min
- Dehydration, embedding (see A.3.2.)

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

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