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Multi-resistant *Escherichia coli* from wildlife

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Summary

Over the past decades multi-resistant Enterobacteriaceae like Extended-spectrum beta-lactamases (ESBL)-producing *Escherichia (E.) coli* have become a major challenge to infection control in both human and veterinary medicine. The spread of antimicrobial resistance occurs mainly through the acquisition of mobile genetic elements like resistance plasmids or the clonal spread of multi-resistant lineages. Most of the resistance genes in pathogens have evolved originally over long periods of evolution in environmental bacteria like the ESBL-enzyme family $bla_{CTX\text{-}M}$ which presumably originates from a soil *Kluyvera* species. Apparently within half a century of usage of antimicrobials in human and veterinarian clinics, the environmental resistome has made its way into bacteria of clinical importance. However, in recent years it has become obvious that we have to consider the other side of the coin of this development as well: the transmission of pathogenic and now multi-resistant bacteria and/or their resistance genes back to the environment and subsequently to wildlife.

My habilitation thesis focuses on multi-resistant *E. coli* as a prototype species for the spread of antimicrobial resistance into wildlife. *E. coli* represents a commensal of the gut of many birds and mammals including humans. Due to its omnipresence in faeces, it is distributed to the environment where it can survive as well. For these reasons it has a long tradition as an indicator bug of faecal pollution. Despite its commensal character, *E. coli* is frequently implicated in intestinal and extra-intestinal infectious diseases, the treatment of which requires the use of anti-infectives. Furthermore, multi-resistant *E. coli,* especially ESBL-producers, are among the “super bugs” which pose a major threat to public health due to limited treatment options in case of infectious diseases. Summing up, this makes *E. coli* an ideal paradigmatic candidate for my research. In contrast to the wealth of studies dealing with ESBL-producing *E. coli* - be it in human medicine, veterinary medicine or livestock breeding - their presence and impact on the microbiota of wildlife has rarely been addressed. Nevertheless, due to the work of a small number of groups including my own, wildlife has gained more attention in recent years, as the occurrence of ESBL-producing *E. coli* in wildlife could implicate consequences like new reservoir functions and transmission pathways, with an impact on human and animal health due to the zoonotic potential of *E. coli*. My initial studies aimed at gaining detailed information on the host distribution of multi-resistant *E. coli* in avian and small mammal wildlife species.

Two avian groups were identified as highly prevalent carriers of multi-resistant *E. coli*, namely birds of prey and waterfowl. But as we also observed multi-resistant *E. coli* in passerines and other avian groups, the carriage does not seem to be restricted, pointing towards the absence of host species dependence. The resistance patterns of the avian isolates are comparable to the ones that have been reported for livestock in Europe. Interestingly, in our study on rural rodents we found lower numbers of multi-resistant *E. coli* compared to rural birds (2% vs. 5%). Nevertheless, similar to the results of the avian study, the resistance pattern of the rodent-borne *E. coli* was comparable to the common antimicrobial resistances observed among *E. coli* from swine, poultry or cattle. Additionally, we found a correlation between antimicrobial resistance of *E. coli* isolates in wild mice and livestock densities for Germany. This corroborates the idea of some kind of environmental pollution with multi-resistant *E. coli* through farming and livestock breeding practises, resulting in higher rates of multi-resistant bacteria in rodents. Our initial screening study on urban rats supported the theory of an influence of synanthropism on carriage rates of multi-resistant bacteria in wildlife, as 13.6% of the rat population carried multi-resistant *E. coli*. The initial screening studies during the first part of my work revealed that a broad range of mammal and avian species can carry multi-resistant *E. coli*. Furthermore, the position of the host in the food chain and its general feeding behaviour seem to be influential factors. Moreover, among the multi-resistant isolates we observed a frequent combination of resistance with phylogenetic backgrounds related to virulence and the possession of genes associated with extra-intestinal virulence. During the second part of my work, I concentrated on ESBL-producing *E. coli*. This enabled a deep characterization and comparison to isolates of human and animal origin. Among the isolates from avian hosts, we detected a clonal lineage of *E. coli* of the sequence type ST648. This sequence type seems to be associated with ESBL-producing *E. coli* in human and veterinary medicine worldwide. Additionally, we described an ESBL-producing *C. freundii* strain from a Tawny owl, which was the first description of an ESBL-producing *Citrobacter* in wildlife.

The detailed characterization of several rat-borne ESBL-producing *E. coli* revealed the presence of a pandemic ESBL-producing *E. coli* lineage with high extraintestinal virulence. At this point in time our work was the first to describe the B2-O25b-ST131 pandemic ESBL-producing *E. coli* lineage in urban rats and, furthermore, in wild mammals in general.

Another ESBL-producing *E. coli* obtained from a rat presented a hybrid strain that paradigmatically combined multi-resistance and high extraintestinal pathogenicity. The isolate belonged to one of the most virulent ExPEC (extraintestinal pathogenic *E. coli*) lineages (ST95) and proved its pathogenicity in an animal infection model. These findings gave initial evidence of the possible role of urban rodents as hosts of ESBL-producing *E. coli* with a high extraintestinal virulence potential. The types of ESBL enzymes as well as the phylogenetic background of the ESBL-producing *E. coli* characterized from wildlife overlap largely with those ESBL enzymes dominant in isolates from human and veterinary patients. As shown for ST131, even identical clonal lineages are present, underlining the zoonotic character of ESBL-producing *E. coli.* In the last part of my work, I used selective isolation methods to verify the carriage rates of ESBL-producing *E. coli* in wildlife. In one study comparing birds of prey from remote and human-influenced areas, we obtained a carriage rate of ESBL-producing *E. coli* of 5 %, independent of where the isolates originated (namely Germany or Mongolia). The Mongolian birds were sampled in the Gobi desert, an area with no considerable human or livestock populations and an absence of agriculture. These findings point towards a contribution of avian migration to the transmission of multi-resistant bacteria in this remote area, as it is very unlikely that the birds picked up the strain locally by the time of sampling.

The characterization of ESBL-producing *E. coli* from birds of prey isolated in Mongolia and Germany, as well as the ones from urban brown rats *(R. norvegicus*), identified clonal lineages that represent clinically relevant isolates with a clear zoonotic potential. In urban rats of the species *R. norvegicus* we furthermore found alarmingly high rates, with of 16% of all animals carrying an ESBL-producing *E. coli*. These rates exceeded those which have recently been reported for healthy individuals from comparable urban settings (5% - 8%), but were similar to the ESBL-producing *E. coli* rates reported in hospitalized patients or their household contacts (12% - 16%). Urban rats might therefore present a sentinel and a possible vector within urban transmission cycles and could become a permanent environmental source of zoonotic and multi-resistant *E. coli*. Both the clear influence of synanthropism as demonstrated by the high rates of ESBL-producing *E. coli* in *R. norvegicus*, and the still substantial carriage rates in remote areas in the birds underlined the need for holistic approaches, comprising humans, animals and the environment to explore putative transmission cycles of multi-resistant ESBL-producing *E. coli*.

In addition, it perfectly depicts the importance of the "One Health" initiative. As a first step in this direction, we comparatively characterized ESBL-producing *E. coli* of avian origin and belonging to phylogenetic group D-and sequence type (ST) 648 producing CTX-M-type ESBLs together with isolates from companion animals, livestock and humans. We were able to prove a pandemic occurrence of the same clonal lineages of D-ST648-ESBL-producing *E. coli* independent from the host. This finding highlights the possibility of interspecies transmission, notably from human to companion and wild animals and vice versa. Besides these public health issues, another dimension should be kept in mind. The widespread occurrence of ESBL-producing *E. coli* in wildlife, even though these animals have never been exposed continuously to antimicrobials, weakens the presumption of a decline of resistance with the absence of antimicrobial selection pressure alone. The theory of a "burden of resistance" might be of overestimated importance, at least for some specific multi-resistant *E. coli* lineages like ST131 or ST648. The frequent observation of a combination of multi-resistance with the possession of genes associated with extraintestinal virulence in the wildlife isolates could be one approach to explaining the success of ESBL-producing *E. coli* in wildlife, and other non-resistance factors might be of equal importance. Based on my data, it seems reasonable that future research in this field should focus on two areas. First of all, detailed epidemiological approaches are needed, as most of the current data is not representative, enabling only preliminary insights. The widespread occurrence of ESBL- producing *E. coli* in wildlife hosts has been proven by my work and the work of others, showing that wildlife might serve as sentinel for the spread of antimicrobial resistance in the environment. Future epidemiological studies should therefore concentrate on holistic approaches either (I) on explicit synanthropic species like rats and possible urban transmission pathways, as the chances of transmission are higher due to the spatial proximity of wildlife and humans or (II) on a global scale on intercontinental migratory birds, as their mobility range and numbers are comparable to international travel. Additionally, it will be important to screen for the arrival of AmpC-and/or carbapenemase-producing *E. coli* in wildlife populations, as their importance in the clinical field is rising and their appearance in the environment seems only a question of time.

Second, we need a detailed molecular characterization of ESBL-producing *E. coli* in wildlife to understand the success of multi-resistant bacteria in non-clinical settings, which might have implications for the treatment of multi-resistant bacteria in the clinics as well.

Studies in this context should (I) verify basic questions of host-pathogen interaction of ESBL-producing *E. coli*, like colonization and shedding and (II) elucidate the influence of non-resistance and/or fitness factors like metabolism, adhesion or motility on the success of ESBL-producing *E. coli* in the environment. As first studies clearly point toward the existence of such contributing factors, my future work will focus on these areas.

Preliminary Note

The work being presented here is based on 12 publications which have been marked in roman numerals throughout the text. All publications have been deposited in the attachments of this work. Unpublished work is implemented in the text as original data.

(I) Guenther S, Grobbel M, Lübke-Becker A, Goedecke A, Friedrich ND, Wieler LH, Ewers C, **Veterinary Microbiology** 2009, 144(1-2): 219-225, Antimicrobial resistance profiles of *E. coli* from common European wild bird species.

(II) Guenther S, Grobbel M, Beutlich J, Guerra B, Ulrich RG, Wieler LH, Ewers C, **Journal of Antimicrobial Chemotherapy** 2010, 65: 582-584, Detection of pandemic B2-025-ST131 *Escherichia coli* harboring the CTX-M-9 extended spectrum ß-lactamase type in a feral urban brown rat (*Rattus norvegicus*).

(III) Ewers C, Grobbel M, Stamm I, Kopp P, Diehl I, Semmler T, Fruth A, Beutlich J, Guerra B, Wieler LH, Guenther S, **Journal of Antimicrobial Chemotherapy** 2010, 65: 651-660, Emergence of human pandemic O25:H4-ST131 CTX-M-15 ESBL producing *Escherichia coli* among companion animals.

(IV) Guenther S, Grobbel M, Heidemanns K, Schlegel M, Ulrich RG, Ewers C, Wieler LH, **Science of the total Environment** 2010, 408(17): 3519-3522, First insights into antimicrobial resistance among faecal *Escherichia coli* strains isolated from small wild mammals in rural areas.

(V) Guenther S, Grobbel M, Beutlich J, Bethe A, Friedrich ND, Goedecke A, Lübke-Becker A, Guerra B, Wieler LH, Ewers C, **Environmental Microbiology Reports** 2010, 2(5): 628–705, CTX –M 15 type extended spectrum beta lactamases producing *E. coli* from wild birds in Germany.

(VI) Ewers C, Bethe A, Wieler LH, Guenther S, Stamm I, Kopp PA, Grobbel M, **International Journal of Antimicrobial Agents** 2011, 37(1): 86-87, Companion animals: a relevant source of extended-spectrum beta-lactamases producing, fluoroquinolone resistant *Citrobacter freundii*.

(VII) Ewers C, Grobbel M, Bethe A, Wieler LH, Guenther S, **Berliner Münchner Tierärztliche Wochenschrift** 2011, 124: 10–17, Extended-spectrum beta-lactamases-producing Gram-negative bacteria in companion animals: action is clearly warranted!

(VIII) Guenther S, Ewers C, Wieler LH, **Frontiers in Antimicrobial Resistance and Chemotherapy** 2011, 2:246: doi 10.3389/fmicb.2011.00246, Extended-spectrum beta-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution?

(IX) Guenther S, Bethe A, Fruth A, Semmler T, Ulrich RG, Wieler LH, Ewers C, **PLoS ONE** 2012, 7(11): e50331, Frequent Combination of Antimicrobial Multiresistance and Extraintestinal Pathogenicity in *Escherichia coli* Isolates from Urban Rats (*Rattus norvegicus*) in Berlin, Germany.

(X) Guenther S, Aschenbrenner K, Stamm I, Bethe A, Semmler T, Stubbe A, Stubbe M, Batsajkhan N, Doi Y, Wieler LH, Ewers C, **PLoS ONE** 2012, 7(12): e53039, Comparable High Rates of Extended-Spectrum-Beta-Lactamase-Producing *Escherichia coli* in Birds of Prey from Germany and Mongolia.

(XI) Guenther S, Wuttke J, Bethe A, Vojtěch J, Schaufler K, Semmler T, Ulrich RG, Wieler LH, Ewers C, **Antimicrobial Agents and Chemotherapy** 2013, 57(5): 2424-2425, Fecal carriage of Extended-spectrum beta-lactamase-producing *E. coli* in urban rats, a risk for public health?

(XII) Ewers C, Bethe A, Grobbel M, Stamm I, Kopp PA, Guerra B, Stubbe M, Doi Y, Zong Z, Kola A, Semmler T, Schaufler K, Fruth A, Wieler LH, Guenther S, **Journal of Antimicrobial Chemotherapy** 2014 69(5): 1224-30, CTX-M-15-D-ST648 *Escherichia coli* from companion animals and horses: another pandemic clone combining multiresistance and extraintestinal virulence?

This habilitation thesis does not contain a material and methods section. All basic methods used are described in publications (I), (II), and (III). Additionally, the results part of my thesis contains a short paragraph describing which methods have been used. Some figures from the original publications are included in the results part. For other figures and detailed tables, please refer to the original manuscripts attached to this work according to the links given in the text.

1. Introduction

The introduction section is separated into five sections starting with general aspects of *Escherichia (E.) coli* and antimicrobial therapy using beta-lactams. This is followed by the current situation of antimicrobial resistance in *E. coli* originating from clinical settings both in human and veterinary medicine and from livestock breeding, thereby focussing on Extended-spectrum beta-lactamases (ESBL). The last two sections summarize the current knowledge on antimicrobial resistance in *E. coli* in non-clinical settings, namely in the environment and wildlife. Throughout this habilitation thesis, ESBL-producers are defined according to the CLSI document M31-A3, therefore on a positive confirmatory test with a difference in the inhibition zones of a 3rd gen. cephalosporin alone and in combination with a beta-lactamase inhibitor like clavulanic acid of $\geq$ 5mm thereby excluding AmpC-producers [1]. The term "multi-resistance" is only used -according to a strict definition in a recent editorial by Schwarz et al. for isolates with resistance to at least three classes of antimicrobials [2]. Throughout this thesis I will distinguish between multi-resistant and ESBL-producing *E. coli*, although ESBL-producing *E. coli* are mostly multi-resistant as well. This is done to underline the fact that ESBL-producing *E. coli* certainly present the next step to pan-resistant isolates and are of utmost clinical importance.

1.1. General Aspects

The most substantial reservoir of multi-resistant Gram-negatives is the gut of man and animals, particularly those who are being treated with antimicrobials[3]. Faecal contamination of water and the environment with these gut-descended multi-resistant bugs is therefore inevitable [3-5]. *E. coli* presents a prototype organism to study this most important route of spread of multi-resistant enteric bacteria, be it from man or animals. This best known member of the Enterobacteriaceae family is an ubiquitous part of the autochthonous intestinal microbiota of mammals and birds. It is found globally, not only in the gut but also in different environments [6-8]. *E. coli* is a Gram-negative, rod-shaped bacterium and hosts can benefit from its presence due to its vitamin K2-production and its capabilities to prevent the establishment of pathogenic bacteria within the intestine [9]. However, *E. coli* is a complex species which can be diversified in the first place into commensals and pathogenic strains [6].

Among the various pathotypes, a general distinction between extra-intestinal (ExPEC) and intestinal pathogenic (InPEC) strains can be made. Both groups harbour a variety of different pathotypes which are displayed in Fig. 1. Most of the *E. coli* isolates characterized during my work present commensals or ExPEC, as ESBL-production in *E. coli* seems to be common in these two groups of *E. coli*. The zoonotic risk of ExPEC has come into the spotlight lately [10-13] and they contribute to infectious diseases of public health concern worldwide, not only as carriers of ESBL-genes. Besides that, InPEC strains have been reported during recent years years as carriers of ESBL-genes as well, and one recent example is the German EHEC outbreak strain of 2011 [14].

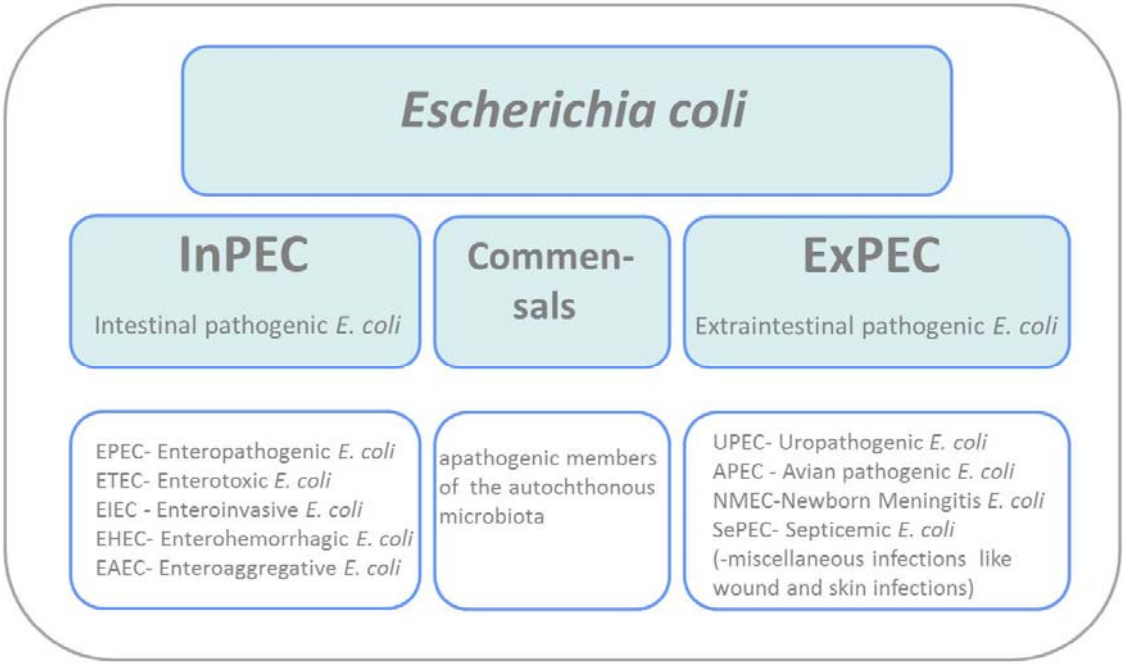


Fig.1: *E. coli* pathotypes

Regarding their phylogeny, *E. coli* are traditionally assigned to four major phylogenetic groups namely A, B1, B2 and D [15]. Although virulence determinants are considered to be mobile, e.g. located on transferable plasmids and PAIs (pathogenicity islands), a link between *E. coli* phylogeny and virulence has been reported widely [15]. Extra-intestinal pathogenic *E. coli* (ExPEC) mainly belong to group B2 and, to a lesser extent, to group D, whereas most commensal strains, which often lack a number of virulence determinants, are affiliated with groups A and B1 [16, 17].

Accordingly, strains from groups B2 and D account for approximately two thirds of all extra-intestinal *E. coli* infections, including urinary tract infection, bacteraemia, meningitis, wound infections, and other miscellaneous infections [18]. The affiliation with the four different phylogroups is based on MLEE (multilocus enzyme electrophoresis) and can mainly be determined via PCR (Polymerase chain reaction) to detect the presence or absence of certain genetic markers [19, 20].

A new approach to determining these phylogroups is based on the bioinfomatic analyses of concatenated sequences of the seven allele sequences gained by MLST (Multi locus sequence typing). By sequence analysis of seven housekeeping gene fragments (a*dk*, *fumC*, *purA*, *recA*, *gyrB*, *icd*, *mdh)* and a Bayesian- based bioinformatic analysis (STRUCTURE) basically the same four major phylogroups can be identified. However, in addition STRUCTURE determined two hybrid groups (AxB1, ABD) which seem to have gained genetic material from different phylogenetic backgrounds [6]. As mentioned above intestinal bacteria like *E. coli* can be easily disseminated in different ecosystems through faeces or faecal pollution of water [3, 4]. This is one of the reasons why this species has been intensively used as an indicator for faecal pollution [21].

Therefore it also seems appropriate to track the spread of antimicrobial resistance into different ecosystems based on *E. coli* [22]. This is emphasized by the fact that *E. coli* is one of the key organisms in terms of antimicrobial resistance in clinical environments. In particular, ESBL-producing *E. coli* are among the so-called “superbugs” like MRSA (Methicillin resistant *Staphylococcus aureus*) or VRE (Vancomycin resistant Enterococci) which pose a worldwide threat to public health [23, 24]. According to the EARS-NET database (http://ecdc.europa.eu/en/activities/surveillance/EARS-Net/database/Pages/database.aspx) the prevalence of clinical cases of MRSA has remained at a high but stable level during recent years, whereas that of ESBL- producing *E. coli* has been continuously rising for the last decade.

In addition to its possible indicator role as a commensal gut bacterium, *E. coli* is frequently implicated in human and animal infections that require treatment by the use of antimicrobials (VII). This adds public health concerns to the list of consequences that arise from the spread of ESBL-producing *E. coli* into wildlife and the environment [25] (**VII**). Although the majority of ESBL-producing *E. coli* is still reported from human clinical isolates [26-28] they are also increasingly recorded in community-acquired bacterial infections.

This indicates that ESBL-producing *E. coli* have been successfully transmitted and now persist in the community [25, 29-31]. The consequences of the spread of ESBL-producing *E. coli* into wildlife and the environment might be comparable to the situation of ESBL in non-clinical human settings. The spread of ESBL-producing *E. coli* in the community presents a new phenomenon which is not fully understood at this point and the occurrence of ESBL-producing *E. coli* in the environment might be a part of the problem. Besides the spread of distinct resistant clones of *E. coli*, the spread of mobile genetic elements carrying resistance genes is also important [32]. As mobile resistance genes can also be transferred between distantly related bacteria originating from any possible source, species that share similar niches draw resistance from similar gene pools [3]. As a consequence of that, other pathogens (especially Gram-negatives) present in the environment might acquire resistance through contact with ESBL-producing *E. coli*.

Nevertheless, this habilitation thesis will mainly focus on the clonal spread of certain ESBL-producing *E. coli* into wildlife and the comparison of these isolates with human and veterinary clinical isolates. Furthermore, this work concentrates on ESBL-production and therefore beta-lactam resistance. This seeming restriction is moderated by the fact that most of the ESBL-producers are multi-resistant. As we define multi-resistance as resistance against at least three classes of antimicrobials [2], the spread of resistances against other classes of antimicrobials is not ignored.

1.2. Antimicrobial therapy and beta-lactam antibiotics

The use of antimicrobial drugs in the treatment of diseases has changed the face of modern medicine [3]. Antimicrobial drugs in human and veterinary medicine are often essential for successful treatment of infectious diseases and a prerequisite for many modern surgical interventions. Since the discovery of penicillin by Alexander Fleming in the 1930s, several new classes of antimicrobial substances with a beta-lactam system or inhibitors of beta-lactamases-like clavulanic acid-have found their way into therapy (Fig. 2, p. 15) [33].

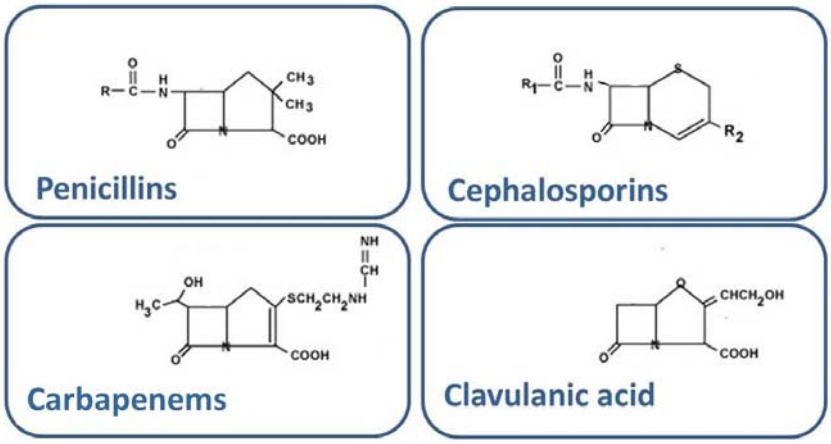


Fig. 2: Molecular structures of beta-lactam antimicrobials

All beta-lactam antibiotics consist of a cyclic amid harbouring two carbon molecules -where the term "beta" comes from-besides the carbonyl moiety and the nitrogen in the hetero ring system (Fig. 2). Besides the diverse class of beta-lactams, other classes of antimicrobials are of utmost importance as well, like aminoglycosides, fluorochinolones, sulfonamides, tetracyclines, lincosamides or peptide-antibiotics, just to mention a few. Despite their long history, beta-lactam antimicrobials are still among the most important antimicrobial agents in human and veterinary medicine [34, 35].

In the veterinary field in Germany alone, the usage of different beta-lactams added up to more than 540 tons, including 3.8 tons of 3rd gen. cephalosporins in 2012 [36]. In human medicine beta-lactams are of equal importance, accounting for approximately one third of all antimicrobial drugs prescribed in Germany [37]. Exact data on the usage of beta-lactams in human medicine is not available, but the total number for all antimicrobial substances has been estimated to be 700-800 tons a year [38, 39]. Even identical chemical compounds are used in both clinical fields. Besides the many different penicillin derivatives, other members of this class have gained a crucial importance in recent decades, namely the first- to fourth-generation cephalosphorins and the beta-lactamase inhibitors like clavulanic acid or the carbapenems as "last line of defense" [40]. The pharmacological principle (Fig. 3, p. 16) of all beta-lactams is identical, as these substances interfere with the final stage of peptidoglycan synthesis through acting on penicillin-binding-proteins (PBP), thereby preventing the bacterial cell wall from being formed.

As the peptidoglycan constitutes a layer between the outer membrane and the cytoplasmic membrane that maintains the cell shape and protects the bacterium against osmotic forces, the interference of beta-lactams with this crucial step leads to the death of growing bacterial cells due to osmotic forces.

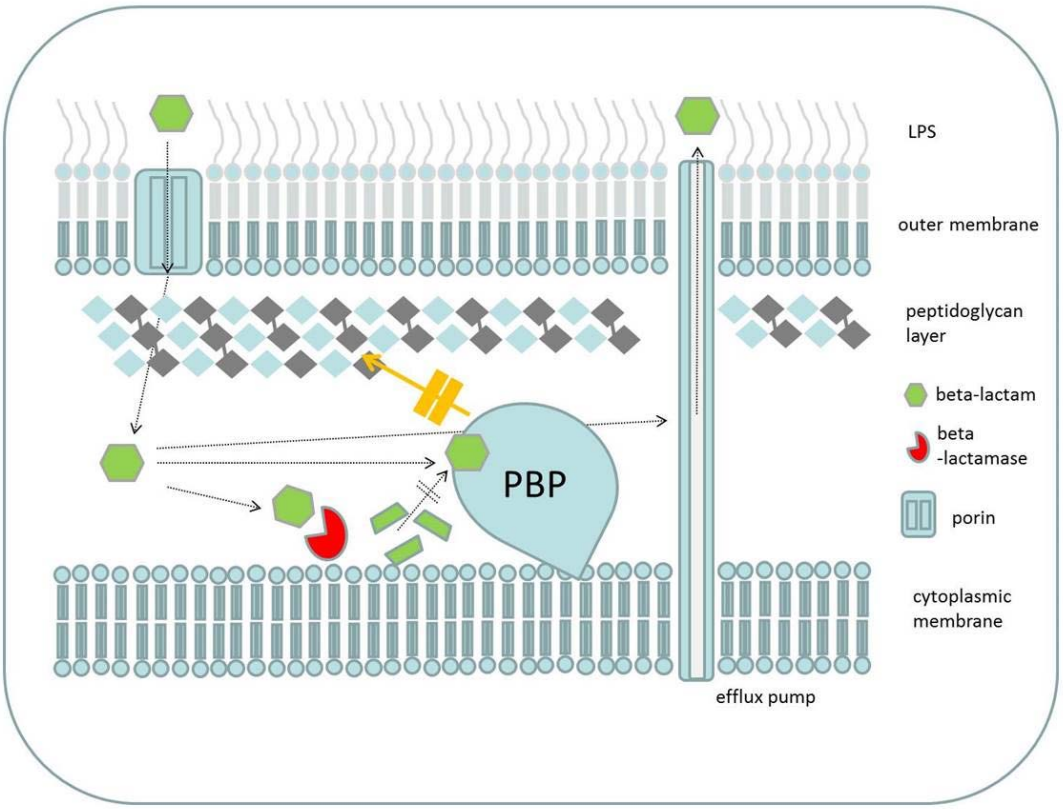


Fig. 3: Pharmacological principle of beta-lactams and their inactivation by beta-lactamases

The first descriptions of resistance appeared shortly after the introduction of beta-lactams on the market in the 1960s [27]. Since then, the prevalence of antimicrobial-resistant strains and resistance genes has changed over the years, and it is impossible to describe this field in detail within this work. Nevertheless, these developments led to the emergence of ESBL-producing *E. coli,* which will be dealt with in the next chapter.

1.3. Extended-spectrum beta-lactamases (ESBL)

Beta-lactamases are bacterial enzymes capable of hydrolysing beta-lactam antimicrobials like penicillins or cephalosporins. The hydrolytic cleavage of the beta-lactam ring system [41] presents the most common resistance mechanism of Enterobacteriaceae spp. against beta-lactam drugs [41]. The cyclic amid of the beta-lactam ring system is hydrolysed and after the opening of the nitrogen hetero cycle, the carbonyl moiety is lost, leaving the remaining structure inactive and not capable of binding to the PBP (Fig. 3 and 4, pp, 16, 17).

Different classification schemes have been described for bacterial beta-lactamases, including the system devised by Bush et al. (1995) which is based on the activity of the beta-lactamases against different beta-lactam antimicrobials leading to four different groups namely 1, 2a, 2b, and 2b' [42]. Currently, the most widely used is the Ambler system, which divides beta-lactamases into four classes (A, B, C, and D) (see Tab. 1, p. 18) based on their amino acid sequences [43]. In the active centre of three of these classes, the amino acid serine is present, whereas for the group B, metal ions are responsible for the catalytic reaction. These enzymes are therefore called metallo-beta-lactamases. Based on these two systems, the majority of ESBLs belong to Ambler class A and to the Bush group 2b.

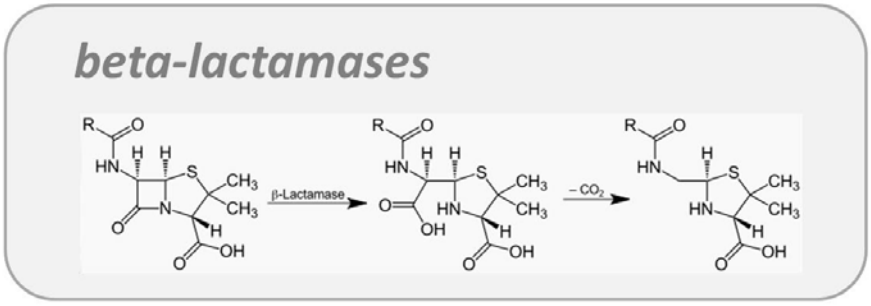


Fig.4: Molecular mechanism of beta-lactamase activity

Currently, a broad variety of more than 400 different beta-lactamase enzymes are known (http://www.lahey.org/studies/). They share the same mechanism of resistance (Fig. 4) but differ largely in their range of substrates and susceptibility to inhibitors. The term “extended-spectrum” describes the capacity to hydrolyse a broader spectrum of beta-lactam antimicrobials than their ancestral beta-lactamases.

These enzymes emerged globally in the late 1990s in Enterobacteriaceae, in particular *E. coli*, but also *Klebsiella* spp. and other members of this bacterial family [44, 45]. ESBLs are capable of inactivating all beta-lactam antimicrobials of the penicillin family (penams) as well as cephalosporins up to the third generation, including substances containing an oxyimino-group such as oxyimino-cephalosporins (e.g. ceftazidime, cefotaxime), as well as oxyimino-monobactams like aztreonam. ESBLs cannot hydrolyse cephamycins and carbapenems, leaving these substances often as the last treatment option.

Inhibitory substances of ESBL are clavulanic acid and tazobactam, which also mark a difference between ESBL- and AmpC-beta-lactamases producing bacteria, as the latter group of enzymes cannot be inhibited by these substances [27]. Currently regarded as the most common ESBLs among Enterobacteriaceae *spp*. are four enzyme families namely TEM (Temoneira), SHV (Sulfhydryl variable), CTX (Cefotaximase)-M and OXA (Oxacillinase) -type beta-lactamase [46].

TEM was first demonstrated in 1965 in an *E. coli* isolate from a patient in Greece, named Temoneira [47, 48]. Today the majority of TEM beta-lactamases are ESBLs.

The first described type of this enzyme (TEM-1) was nevertheless only able to hydrolyse penicillin derivatives and is thus not regarded as ESBL. The same is true for TEM-2 and TEM-13 [49]. At present, this group of beta-lactamases consists of more than 150 different enzymes (http://www.lahey.org/studies/).

Similar to the situation in the TEM beta-lactamase family, the majority of SHV enzymes are ESBLs as well. SHV enzymes are derivatives of SHV-1, which merely confers resistance to broad-spectrum penicillins, and SHV-2, which is able to hydrolyse cefotaxime [48]. Both enzymes were described first in the 1980s in Europe [48].

In contrast to the TEM- and SHV-type beta-lactamases families, the situation for the OXA-type beta-lactamase is complex. Most enzymes in the family are not regarded as ESBLs because they do not hydrolyse third generation cephalosporins (with the exception of OXA-10, OXA-2, and their derivatives [http://www.lahey.org/studies/]). However, distinct OXA-types (like OXA-48) even exceed the hydrolysing properties of other ESBLs by being carbapenemases and therefore are able to hydrolyse penems like imipenem as well. They haven't reached the importance of ESBLs in Enterobacteriaceae yet, but play an important role in antimicrobial resistance, e.g. of *Acinetobacter baumannii* [50].

Serin beta lactamases						Metallo beta-lactamases
Ambler class	**A**		**C**	**D**		**B**
Designation	ESBL	Carba - penemase	AmpC	OXA		Metallo beta-lactamases
Examples	CTX-M TEM SHV	KPC GES	AmpC CMY MOX DHA	OXA type ESBL	OXA type carbapenemases	VIM NDM IMP
Occurrence*	Enterobacteriaceae		Entero-bacteriaceae	Enterobacteriaceae *A.baumanii*		Enterobacteriaceae *A.baumanii* *P.aeruginosa*
Phenotypic resistance	penicillins cefotaxime	penicillins cephalosporins carbapenems	penicillins cefotaxim	*oxacillin* *cefotaxime*	penicillins cephalosporins carbapenems	penicillins cephalosporins carbapenems

Tab.1: Classification of beta-lactamases based on the Ambler system * preferred but not restricted

CTX-M-type beta-lactamases are currently regarded as the most important ESBL-enzyme family, named after their ability to hydrolyse cefotaxime. The initial isolation was from clinical samples in the early 90s in Munich, where the abbreviation CTX-M comes from [51]. Comparison

to environmental beta-lactamases from soil organisms revealed their close relationship to beta-lactamases from *Kluyvera* spp. [52].The CTX-M family currently comprises more than 70 different enzymes divided into five groups depending on their amino acid sequence (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25) [28].

Besides these classical ESBLs, other beta-lactamases with an extended-spectrum of hydrolysing activity have become very important over the last two decades as well. The inconsistent group of *ampC* beta-lactamases confers resistance to most beta-lactam antimicrobials, with the exception of methoxy-imino-cephalosporins (cefepime) and carbapenems [47]. In contrast to ESBLs they are not inactivated by beta-lactamase inhibitors like clavulanic acid. The rise of carbapenem hydrolysing beta-lactamases like New-Dehli-Metallo-beta-lactamase (NDM)-1 or OXA-48 in recent years marks a development that might inevitably lead to pan-resistant Enterobacteriaceae strains. Clearly carbapenemases threaten the clinical utility of this antibiotic class, which often act the last treatment option [50, 53]. Carbapenemases are beta-lactamases that not only inactivate oxyimino-cephalosporins and cephamycins but are also capable of hydrolysing carbapenems like imipenem or meropenem. This group of beta-lactamases is very diverse and can be found in three different β-lactamase classes (class A, B and D). ESBLs have been found in a wide range of Gram-negative bacteria, but the majority of bacterial hosts belong to the family of Enterobacteriaceae, including *Klebsiella* spp., *E. coli*, *Salmonella enterica enterica*, *Citrobacter* spp., and *Enterobacter* spp. [27].

1.3.1. ESBLs in human and veterinary medicine

Initially ESBL-producing Enterobacteriaceae were only observed in clinical settings, with the first nosocomial outbreaks of ESBLs recorded in clinics in central Europe in the late 1980s [51, 54]. Since then an explosive worldwide dissemination of ESBLs in human clinical settings has taken place [24-28, 45, 46, 50, 55-59]. Although the first ESBL-encoding gene ever identified was a member of the $bla_{\text{CTX-M}}$ family, namely a $bla_{\text{CTX-M-1}}$ producing *Klebsiella pneumoniae* strain [51], early human ESBL-producing isolates from this period mainly presented TEM and SHV beta-lactamases. This past dominance of bla_{SHV} and bla_{TEM} genes has been replaced since the year 2000 by the rapidly growing family of $bla_{\text{CTX-M}}$ in human clinical and community settings [26, 60, 61]. The prevalence of classical ESBL-enzymes like TEM or SHV has been decreasing steadily since then [58].

Among the CTX-M enzymes apparent in *E. coli*, CTX-M-15 seems to be of utmost importance. Within the last decade CTX-M-15-producing *E. coli* have emerged and disseminated worldwide as an important cause of both nosocomial and community-onset urinary tract and bloodstream infections in humans [23, 24, 28, 62].

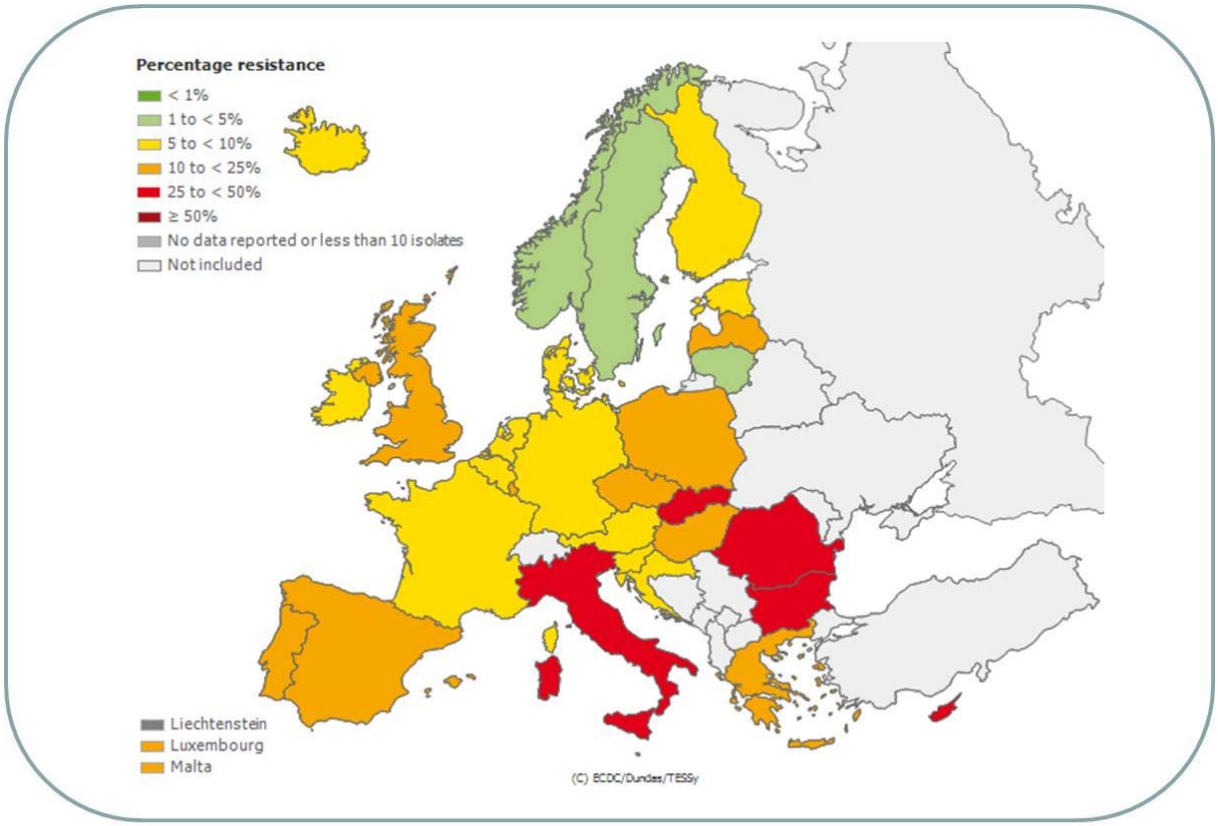


Fig.5: Human clinical *E.coli* isolates in Europe resistant to 3rd gen. Cephalosporins according to the ECDC Tessydatabase (data from 2012)

This sudden pandemic increase of CTX-M-15-producing *E. coli* has been largely influenced by the spread of one single clonal group, namely B2:O25b:H4-ST131-CTX-M-15, across different continents [63, 64].

Fig. 5 (source: http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/database.aspx) displays the percentages of invasive *E. coli* from human clinical samples that are resistant to 3rd generation cephalosporins from different European countries. Most of these clinical isolates can be regarded as ESBL-producers, and although the rates differ from country to country, for most parts of central Europe more than 10% of clinical *E. coli* isolates are ESBL-producers. In Europe, ESBL-producing Enterobacteriaceae have been spread at an alarming rate. Although there is a large difference between European countries, almost every European country has experienced outbreaks with ESBL-producing organisms [65].

Rates of ESBL-producing *E. coli* in South America and Asia rank amongst the highest in the world, with 8.5% to 18% *E. coli* isolates being ESBL-producers. Rates for ESBL-producing *E. coli* in the United States are rather low compared with the rest of the world, at approx. 2 %. Although there is a general lack of comprehensive data regarding ESBL-producing Enterobacteriaceae in Africa, there is sufficient evidence for a high prevalence of ESBLs in African countries [59].

As an example, Egypt has an extremely high rate of ESBL-producers, with up to 70% of isolates producing the enzyme [65]. ESBLs are therefore a worrying global public health issue as infections caused by such enzyme-producing organisms are associated with a higher morbidity and mortality and greater fiscal burden [65]. The worldwide increasing prevalence of ESBLs and an ever diminishing supply in the antibiotic armamentarium represent a clear and present danger to public health [65].

In the veterinary field, several studies reported the occurrence of ESBL-producing *E. coli* originating from food-producing and companion animals within the last ten years [66, 67] **(VII)**. These ESBL-producing *E. coli* did not only present resistant commensals but also include isolates relevant to human health, as they belonged to the ExPEC pathotype [68-72]. Companion animals were the first to be reported with ESBL-producing *E. coli* in veterinary medicine. An SHV-12-type beta-lactamase-producing *E. coli* was the first clinical ESBL-producing bacterium isolated from a dog in Spain in 1998 [73]. These reports were rather sporadic but as in the human field TEM and SHV were the predominating types detected in isolates from dogs in Italy and Portugal [74, 75]. The changing epidemiology of ESBL-types towards the dominance of CTX-M-enzymes that was observed in human medicine might be mirrored in small animal medicine, and recently several studies also reported instances of CTX-M-producing *E. coli* originating from companion animals. Several authors therefore assume that a leading role for CTX-M type enzymes in future resistance will be problematic in veterinary medicine [69, 71, 76, 77]. The recent emergence of the human pandemic clonal group B2-O25b:H4-ST131-CTX-M-15 in companion animals underlines this hypothesis [25, 72, 78, 79]. Over the last decade the number of publications reporting ESBL-producing *E. coli* in livestock has increased[80, 81]. Similar to the case in companion animals, most of the ESBL-enzymes identified in *E. coli* from livestock are present in bacteria from humans as well [69].

As reviewed by Ewers et al. (2012) the most common genes associated with ESBL-production were various CTX-M types (*bla*$_{CTX\text{-}M\text{-}1,15,9}$), followed by *bla*$_{TEM\text{-}52}$ and *bla*$_{SHV\text{-}12}$, with other TEM and SHV types also observed [47, 69]. The earliest description of poultry as a carrier of ESBLs was reported in 2003 by Brinas et al., who observed CTX-M-14 and SHV-12-producing *E. coli* in faecal samples of healthy chickens in Spain [82]. Enterobacteria-carrying CTX-M-14 and CTX-M-2 were observed in healthy poultry sampled in Japan during the same period [83, 84]. Soon after that, studies documenting the occurrence of various ESBL-types isolated from pigs and cattle followed [85].

Among food-producing animals, poultry seems to be of major importance. Several studies focused on this group of animals, which might initially have been influenced due to the fact that poultry and its products present a known infection source of food-borne zoonotic pathogens, including *Campylobacter* spp. and *Salmonella enterica* ssp. *enterica* serovars. Larger datasets on poultry are available for Europe and Asia, and studies report detection rates up to 44 % of ESBL- or AmpC-producing *E. coli* and *Salmonella* [47]. Data originating from the Americas and Africa is limited but establishes the global occurrence of ESBL-producing bacteria in poultry [47]. Even though poultry seems to be the most potent animal species hosting ESBL-producing *E. coli*, other food-producing animals, including cattle, pigs, and sheep, are also colonized or infected by such bacteria. ESBL-producing *E. coli* have been reported in cattle and pigs all over the world. The ESBL-enzymes involved varied a lot between the studies and different geographic origins. As an example, CTX-M-1 is clearly the dominating enzyme in cattle and pigs in Europe, with 72% of studies reporting this enzyme, whereas in Asia the situation seems to be much more diverse with CTX-M-15, CTX-M-14, CTX-M-2 and other CTX-M enzymes involved [47].

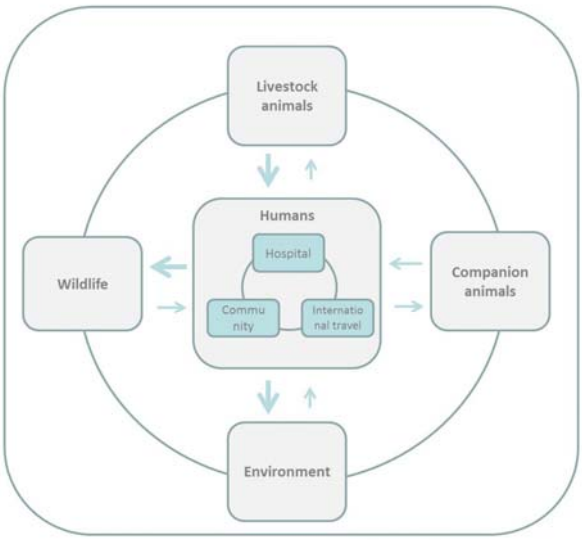


Fig.6: Simplified transmission pathways scheme of ESBL-*E. coli* (modified from Ewers et al. 2012)

The detection rates of ESBL-producing *E. coli* varied a lot between the different

livestock host species as for cattle the rates reported span from 0.4% to 17% [47] and for pigs studies observed 0.2% up to nearly 30%.

1.3.2. Zoonotic aspects of ESBL- producing *E. coli*

Rising numbers of ESBL-producing *E. coli* in food-producing and companion animals [69, 71] lead to the hypothesis that both groups of animals might turn into infection sources or even reservoirs--the natural persistent source of infection--contributing to the spread of these bacteria into the environment and also to humans. Fig. 6 depicts possible transmission scenarios of ESBL-producing *E. coli* in between different hosts and environments. As the natural habitat of *E. coli* is the mammalian gut, livestock especially shed these bacteria, which might lead to a massive faecal spread via manure [86]. In this context the onset of ESBL-producing *E. coli* isolated from food like vegetables should be kept in mind as well [87].

For companion animals other aspects of transmission seem to be important. The role of companion animals has become more similar to that of full family members and they share intimate contacts with their owners, which can result in transmission cycles of multi-resistant bacteria, as it is known that the microbiota of pets and their owners can be very similar [25, 72, 88, 89]. Nevertheless, based on the data that currently exists on types of ESBL-enzymes and the phylogenetic background of the multi-resistant isolates from different animal sources and humans, it is impossible to determine the impact of ESBL-producing bacteria of animal offspring on public health. One first step is the estimation of the occurrence of these isolates in different animal species, which is currently performed for Germany in great detail in the BMBF Network RESET (http://www.reset-verbund.de). This has also been done on a smaller scale in several studies and was followed by a detailed characterization of the ESBL-enzymes involved, as reviewed by Ewers et al. [47]. A central issue in the complex transmission scenarios is sharing of identical beta-lactamase types and certain bacterial clones with an identical phylogenetic background by human and domestic animal hosts. Apart from common ancestry of ESBL-producing bacteria and the types of ESBL-enzymes, the sharing of identical plasmid types by human and animal hosts is important. A detailed summary on the occurrence of plasmid types carrying ESBL-genes in bacteria from animals and human was published recently [90, 91].

The role of these different influences is exemplified by the global success of, e.g., CTX-M-type ESBLs, which is due to the wide dissemination of particular plasmids, ESBL-enzymes and bacterial clones [45].

Regarding the overlaps of the different types of ESBL-enzymes in human and animal bacteria, Ewers et al. (2012) reported CTX-M-1, CTX-M-14, CTX-M-15, and SHV-12 as the most common types in companion and livestock animals all over the world. In this analysis of the current ESBL-data available, a similar distribution of ESBLs was also shown for humans, as CTX-M-14 and CTX-M-15 were the major types regardless of their geographic origin [47]. Ewers et al. (2012) also reported the dominance of CTX-M-1 among animal isolates from Europe, including companion animals, poultry, cattle and pigs.

Interestingly, CTX-M-1 is reported in lower abundances from human sources in Europe, thereby calling the impact of livestock ESBL on human public health [47] into question.

However, two studies from the Netherlands recently identified CTX-M-1 as the most prevalent ESBL-type shared by human patients, healthy carriers, poultry, and retail chicken meat, suggesting a recent cross between human and avian hosts [68, 92]. The authors suggested a relationship between contamination of chicken meat and appearance of ESBL-genes in *E. coli* from humans and thus transmission of ESBL-producing *E. coli* from poultry to humans. The authors also stated that this could not be proven unequivocally, as the phylogenetic background of the ESBL-producing isolates from both sources presented a highly frequent type of *E. coli,* namely ST10. Whole genome sequence data of these isolates would therefore help to ascertain epidemiological links between these two groups of hosts. Additionally, case control studies are necessary to prove possible transmission scenarios. In Asia CTX-M-14 is among the most prevalent beta-lactamase types recovered from companion animals and poultry and, to a lesser extent from cattle and pigs.

Although CTX-M-15, which has spread worldwide in human isolates [45], has been reported to occur worldwide in isolates from livestock and companion animals, it seems to be not among the major enzymes detected in livestock animals where it has been found only incidentally in poultry in European countries and on a small scale in cattle and pigs. Nevertheless in companion animals it has become an important subtype of ESBL as well. The observed patterns of the different types of beta-lactamases are highly diverse and incongruent in livestock and companion animals all over the world.

In contrast to that, the distribution of different types of ESBL in humans is comparable all over the world, indicating the importance of human-to-human contact for the transmission of ESBL-producing strains [47].

There has been a massive public discussion in the press, on television and in the community in the last two years about the impact especially of livestock-originating ESBL-producing *E. coli* on clinical ESBL cases, painting a rather mono-dimensional picture of a transfer from livestock to humans. This theory is not backed up by scientific evidence, which would allow us to determine the main direction of transmission of ESBL-producing bacteria between human and animals in general. There is only limited evidence for the spread of ESBL-producing organisms via direct contact with livestock, and in one study people working with poultry seemed to have a higher risk for intestinal carriage of ESBL-producing bacteria [91]. Partial overlap of beta-lactamase types from human ESBL-producing *E. coli* with those of animal origin favours the idea of an on-going inter-host transmission of multi-resistant strains. Nevertheless, the identification of the genetic background --and therefore the phylogeny of the strains --is a key issue in untangling the complex interplay of transmission and the putative zoonotic nature of animal-derived strains. A common and powerful tool for these purposes is Multilocus sequence typing (MLST). Although based on a small set of seven marker genes only (*adk*, *fumC*, *purA*, *recA*, *gyrB*, *icd*, and *mdh*), it reflects the microevolution of the *E. coli* core genome, thereby providing a sketch of the population structure of bacteria [6,93]. MLST is a system in which, based on allele combinations of seven housekeeping gene fragments, a sequence type (ST) is determined. At present, more than 3.000 STs have been submitted to a public database **(**www.mlst.net). The phylogenetic relationships of different STs can be visualized via Minimum spanning trees (MSTrees). Fig. 7 (p. 26) presents such an MSTree based on 7,766 *Escherichia coli* isolates which were analysed based on publicly available data by Ewers et al. [47]. In recent years, data on the phylogenetic background of ESBL-producing *E. coli* has become more and more available. Nevertheless, none of these datasets can really determine the directions of transmission between human and animal sources. Recently, several STs have been identified among ESBL-producing *E. coli* which seem to be linked to ESBL-production.

Various ancestral groups are involved such as group D (STC38, ST405, and STC69), group A (ST10, ST167, and ST617), group B1 (ST410), or hybrid group ABD (ST648) and of course most prominently ST131 of group B2.

For most of these sequence types, a global occurrence in companion animals, livestock and humans can be observed. In other words, ESBL of the same phylogenetic background occur in domestic animals and humans. The most prominent example of this situation is the recognition of one particular pandemic ESBL-producing clone, namely B2-O25b:H4-CTX-M-15 of ST131 in humans [63], livestock, companion animals and wildlife (**VIII**).

This pandemic strain presents a clonal group of high extra-intestinal virulence, causing urinary tract infections, bacteraemia, urinary sepsis, and neonatal sepsis in humans [63, 64]. The affiliation with the identical phylotype ST131 and the partial overlap between human and poultry *E. coli* beta-lactamase genes (e.g. $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$) suggested a transfer of such strains to humans via consumption of poultry meat as a plausible, but still unconfirmed scenario [68, 92].

Besides livestock, companion animals have also been identified as carriers of ST131 and we recently confirmed the presence of CTX-M-15- and SHV-12-producing ST131 *E. coli* in dogs, cats and one horse, accounting for 6% of all ESBL-producing *E. coli* isolates typed (**III**).

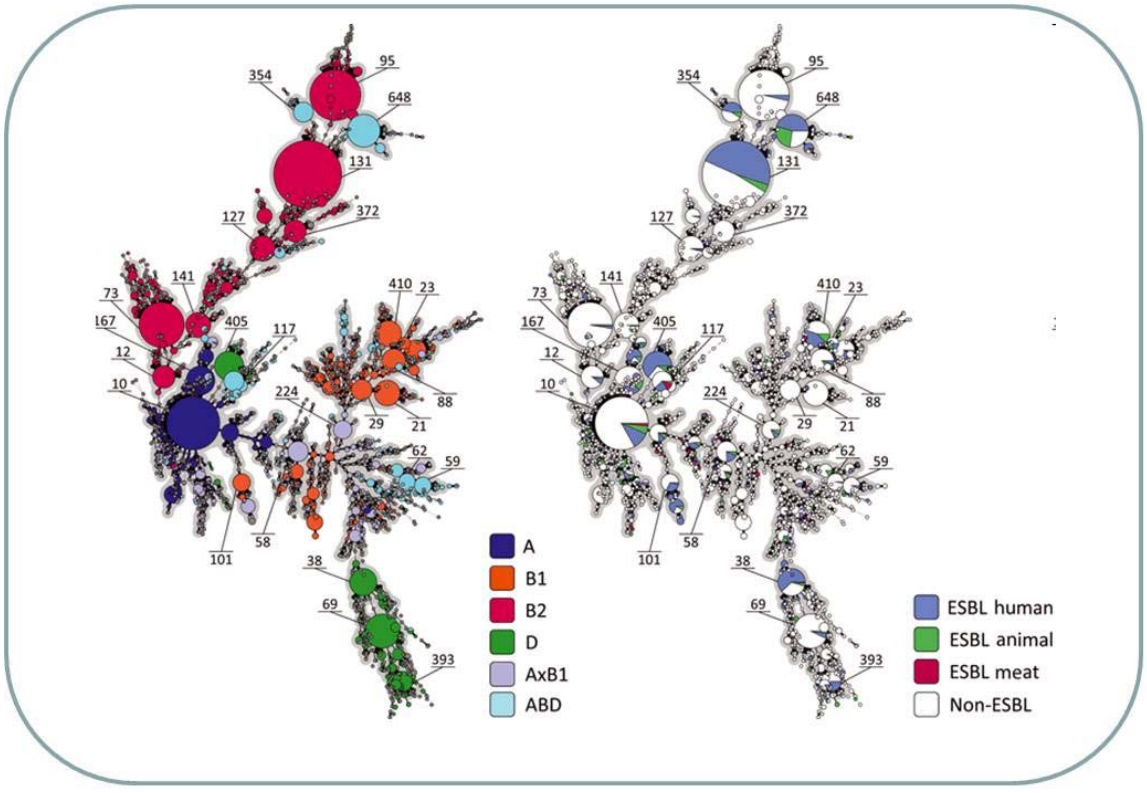


Fig. 7: Minimum spanning tree of the population structure of 7766 *E. coli* strains (Ewers et al. 2012) Left panel: distribution of phylogenetic/ ancestral groups based on Structure analysis. Right panel: distribution of ESBL/AmpC/NDM-1-producing *E. coli* in the background of the *E. coli* population.

1.4. Antimicrobial resistance in the environment

With only some exceptions, most of the antimicrobial substances currently used in human and veterinary medicine have natural ancestors produced by soil bacteria like *Streptomycetes* or fungi like *Penicillium* spp. [94]. The occurrence of antimicrobial resistance and the respective resistance genes in the environment is therefore an ancient phenomenon, as these producers must display intrinsic resistance to the antimicrobial compound they produce themselves [94].

The original functions of these compounds were presumably more related to microbial competition for an ecological niche or related to *quorum sensing*, the prokaryotic system of stimulus and response correlated to population density [94].

With the adoption and derivatization of these compounds in modern medicine, their role has changed towards the "weapon-shield" function they play in clinical settings today [4, 95, 96]. The increase of non-intrinsic antimicrobial resistance in pathogenic bacteria started after the introduction of antibiotics in medicine some 60 years ago suggesting a correlation between antimicrobial pressure and the emergence of resistance in pathogens [96, 97]. It seems that the selection pressure on pathogenic bacteria resulting from the massive medical and veterinary use of antimicrobials in a short time led to a transmission of the environmental resistome to clinical pathogens [4, 95, 96]. However, it is obvious that this transmission is not mono-directional and we might currently face the reverse phenomenon, namely a massive spread of resistance genes, resistant bacteria and in addition antimicrobials into the environment.

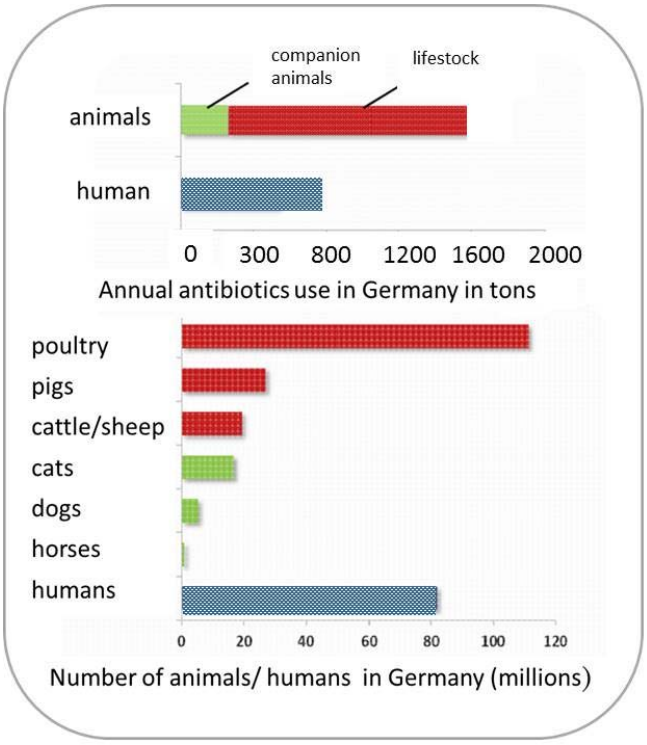


Fig.8: Antibiotic use in Germany in 2012 and numbers of animals/humans

Although hard to measure, the scale of this flow of genes, bacteria and antimicrobials surely exceeds the original impact from the environment to the clinics. The substantial use of

antimicrobials (Fig. 8, p 27) in human medicine (Germany: 700-800 tons per year [2012] [38, 39]) as well as in livestock breeding (Germany: 1,700 tons per year [2012] [39]) has led to a constant release of antimicrobials into the environment [95, 98-100]. After their admission to the body of human or animal patients the antimicrobial compound will be--at least--partly-metabolized. Metabolites--partly still active--and the original antimicrobial compound will then be secreted via urine or faeces. As an example, after the admission of beta-lactams, up to 30% of the original compound is secreted on the renal way without any metabolic changes. As a result of that, metabolites and non-metabolized antimicrobial compound will be emitted to the sewerage system [101].

Depending on the chemical properties of the substance (like polarity or water solubility), the compound might be degraded, or will persist in the sewage sludge, or is directly released into surface waters [102]. If the sludge is used as a fertilizer, sludge-associated drugs will enter agricultural systems as well [102]. On the global scale, the presence of sewage systems and controlled wastewater disposal and management is not a common standard. Antimicrobials can therefore also reach agricultural soils directly through irrigation with wastewaters and surface waters [4, 102]. This is also the case for some antimicrobials used in agriculture and as growth promoters in livestock breeding in some parts of the world. They can be released into the environment either directly, from use in aquaculture, or indirectly during the application of manure and slurry from intensive livestock facilities [3]. Once released to the soil system, compounds can be transported to surface water or groundwater and start cycling within the environment [103, 104]. This can lead to unforeseeable impacts on the environmental microbiota as antibiotic-producing bacteria occur naturally throughout the environment and their complex interplay is then influenced by mixing with exogenous bacteria from anthropogenic sources [105].

The pollution of the environment with antimicrobial residues and exogenous resistant bacteria provides the ideal selective and ecological conditions for melting pots and hotspots for horizontal gene transfer, causing new resistant strains to arise [106]. Besides that, the constant release of antimicrobial compounds into the environment might also lead to a sub-inhibitory antibiotic pressure which in turn might promote the persistence of pathogenic multi-resistant bacteria in the environment [96].

Some semisynthetic antimicrobials can persist in soils for long periods of time at high concentrations [107] and most of the clinically important antibiotics like fluorochinolones,

sulfonamides, tetracyclines and macrolides have been detected in soils, surface water, sediments, and groundwater [108, 109]. The reported concentrations of antimicrobials were rather low, but even these concentrations are selective for antimicrobial resistance [110-112]. Antimicrobial substances have been recorded throughout the year across various hydrological, climatic and land-use settings, and tetracyclines as well as fluorochinolones even persisted in the environment for months to years [113, 114]. This is of special critical importance in terms of multi-resistant ESBL-producers, as the co-resistance to these substances is often found in ESBL-producing *E. coli*.

Besides the pollution of the environment with the antimicrobial compounds, there is also substantial evidence that the transmission of mobile genetic resistance elements and multi-resistant bacteria from anthropogenic sources is of highest importance.

Several studies have shown that during sewage treatment, ESBL-producing Enterobacteriaceae were present at all stages of sewage treatment [115] or even at the outflow of wastewater treatment plants as shown in the Czech Republic and Ireland [116, 117]. Mobile genetic elements carrying genes conferring resistance to beta-lactams and aminoglycosides have been isolated from activated sludge in Germany and Portugal, proving the survival of such genes until the final stage of wastewater treatment [118, 119]. Besides human sewage, the transmission of resistant bacteria and resistance genes into the environment via manure has been addressed in several studies as well. The amount of manure from livestock spread on the field has been estimated at 10 million tons per year [120]. Besides that, studies on livestock farms in Germany recently showed a direct pollution of the close proximity of these farms with MRSA and ESBL-producing bacteria [80]. As discussed earlier, ESBL-producing *E. coli* are highly prevalent in livestock breeding, consequently leading to a dissemination of these bacteria on agricultural soil. Hartmann et al. (2012) even showed a survival of such ESBL-producing isolates on soil for more than one year [121]. Additionally, multi-resistant Gram-negatives were detected in subsurface flow several months after pig slurry was applied to agricultural soils, pointing toward their persistence and dissemination to water [122]. Pig slurry has been additionally reported to carry antimicrobial resistant *E. coli* depending on the occurrence of heavy metals [86].

1.4.1. Antimicrobial resistant bacteria in wildlife

The common occurrence of multi-resistant bacteria in wildlife presents a relatively new phenomenon. Nevertheless, it could have been anticipated, as antimicrobial resistant

pathogenic bacteria were already found in environmental samples apparently free of any antimicrobial pressure decades ago [123-126]. Moreover, as *E. coli* is ubiquitous and asymptomatically colonizes the gut of birds and mammals, its spread in the environment is almost inevitable [6-8]. The dynamics and nature of the spread of ESBL-producing *E. coli* into the environment (plasmids vs. clones) and subsequently into wildlife are influenced by general aspects of the *E. coli* population. The use of antimicrobials selects for resistant clones, with one mechanism being horizontal gene transfer between strains [127]. It seems unlikely that pathogens isolated from wildlife have acquired resistance through new parallel mutations in the respective genes due to sub-inhibitory concentrations in the environment.

Horizontal transfer of resistance genes from clinical isolates or the intake of already resistant bacteria from human waste, sewage and domesticated animal manure seems much more likely [4, 111]. Both explanatory models seem appropriate, but the current data allows no quantifications of the importance of either way, as details of the spread of multi-resistant-*E. coli* into natural environments are far from being characterized. Seen from a historical perspective, the first reports on antimicrobial resistance for single antimicrobials in *E. coli* from human and animal populations lacking selective antimicrobial pressure date back to the 1960s [128]. The first antimicrobial resistant *E. coli* isolates originating from wildlife were reported at the beginning of the 1980s from wild birds in Japan [123-125]. Five years later, baboons feeding on human waste in South Africa were reported as carriers of resistant *E. coli* as well [129, 130]. While these older studies are far from being representative in any way, they have in common that the number of single resistances was low and multi-resistant isolates were only seldom present. The situation changed a decade ago with an increase in the number of studies describing the occurrence of antimicrobial resistant *E. coli* in wildlife [71, 131-146]. In some of these studies on wildlife, the first multi-resistant *E. coli* isolates were detected [2, 71, 131-146]. The first description of an ESBL-producing *E. coli* of wildlife origin dates back to eight years only [147] and since then several reports followed [69, 148-167].

Research Issue

In the beginning of my research on *E. coli* in wildlife we focussed on the occurrence of pathogenic *E. coli* in wild birds. During one of these studies we detected multi-resistant *E. coli* in Mallard ducks (*A. platyrhynchos*) [168]**.** At this point in time (2008) very little was known about antimicrobial-resistant bacteria in wildlife. Taking into consideration the growing concerns about infections with multi-resistant bacteria in humans as well as the increasing evidence of new zoonotic pathogens spilling over from wildlife to humans [169, 170], it seemed important to gain knowledge on the situation in wildlife. Therefore, I focussed on

Different avian and mammal species as hosts

of antimicrobial resistant *E. coli* first. The following principle questions were at that time unsolved and the first part of my work aimed at gathering basic knowledge: Which avian and mammals host species are carriers of multi-resistant bacteria? Is there a host species dependence of multi-resistant bacteria? Which role does synanthropic behaviour of the host play? Is there a differential spatial distribution of these multi-resistant bacteria?

As we had already detected multi-resistant *E. coli* in Mallard ducks, we consequentially included a diverse set of birds in an initial screening study **(I)**. As some of the first reports on multi-resistant bacteria in wildlife dealt with small mammals and rodents, we included in a second attempt rodents and small mammals from rural areas and furthermore urban rats **(IV, IX)**. This host species distribution was chosen to answer some of the questions mentioned above, but it was also influenced by the availability of samples. As we indeed detected ESBL-producing *E. coli* in wild birds and rats during these studies, my following work concentrated on

ESBL-producing *E. coli* in wildlife

and the basic questions concerning the occurrence of ESBL-producing *E. coli.* They were partly the same as the ones mentioned above, such as the host species distribution, the role of synanthropism and host ecology. Nevertheless, the main questions during this part of the work were: Which types of ESBL enzymes are present in wildlife?

Which additional resistances are carried by ESBL-producing *E. coli* from wildlife? What is the phylogenetic background of the ESBL-producing *E. coli* found in wildlife? Is the resistance pattern comparable between ESBL- producing *E. coli* from human, livestock or companion animal origin?

To answer some of these questions we characterized ESBL-producing *E. coli* isolates from different wildlife origins in detail, including phenotypic and genotypic resistance screening and characterization of the phylogenetic background. We also compared wildlife *E. coli* to ESBL-producing *E. coli* originating from humans, livestock and companion animals **(II, III, V, VI, VII)**. As the detailed characterization of ESBL-producing *E. coli* from wildlife revealed that basically the same types or even clones of ESBL-producing *E. coli* are present in wildlife and in the clinical field, I further on focused my research on some of the implications of these findings on

Carriage rates, pathogenicity and zoonotic potential of ESBL-producing *E. coli* from wildlife

The detection of ESBL-producing *E. coli* in wildlife indicates possible environmental consequences and leads to important questions like: What are the rates of carriage of ESBL-producing *E. coli* in birds? Can ESBL-producing *E. coli* spread via bird migration? The occurrence of zoonotic ESBL-producing *E. coli* in urban rats consequently leads to questions like: How high are the rates of ESBL-producing *E. coli* in rats? How pathogenic are ESBL-producing *E. coli* from wildlife? Can rats spread ESBL-producing *E. coli* out of the sewage system back to the human infrastructure? To contribute to background information for some of these problems we therefore continued the screening of urban rats, now also including selective screening for ESBL-producing *E. coli*, and sampling rats directly from the sewage system nearby the sewage outflow of a large university clinic **(XI**). An infection experiment was performed comparing ESBL-producing isolates with typical ExPEC-strains. We furthermore sampled birds of prey from Germany and a remote area in Mongolia for ESBL-producing *E. coli* **(X)** to help to verify some of the questions mentioned above. Finally, as we detected a clonal group of ESBL producing *E. coli* of ST648 in two of our wildlife studies, we investigated the occurrence of this clonal group in veterinary and human clinical samples and compared clones of this group in detail (**XII**) to demonstrate the zoonotic character of ESBL-producing *E. coli*.

2. Results

The results section of this work is based on 12 publications listed in the preliminary note and presents a short overview of the main results of every study (Fig. 9). For detailed data please refer to the attached original manuscripts. Some unpublished results are included as original data in the text. There are three main chapters, which present the results of my past work.

1. **Avian and mammal host species carrying multi-resistant *E. coli***
2. **Extended-spectrum beta-lactamases-producing *E. coli* in wildlife**
3. **Carriage rates, pathogenicity and zoonotic potential of ESBL-producing *E. coli* from wildlife**

In the heading of each subchapter, the number of the publication from which the data originates is listed. For reasons of readability and clarity of the results section, the following definitions of isolates and clones have been used. Each *E. coli* colony was regarded as an individual isolate. A clone was defined as an *E. coli* group of isolates with a specific macro-restriction pattern, whereas two clones differed by more than one band [171]. The term strain is used generally for characterized lineages of *E. coli*. In most of the publications we used a standard set of phenotypic and molecular genotypic screening methods which will be shortly mentioned hereafter. For further details please refer to the original publications. If, besides the standard set, other methods were applied, they will be mentioned in the subchapters. A table is attached at the end of the results section summing up the name and number of the host species tested, number of isolates screened, clones defined and rates of multi-resistant and/or ESBL-producing *E. coli.*

All *E. coli* isolates of my studies were screened by agar diffusion test according to the CLSI criteria [1] for phenotypic resistance against important antimicrobial substances from human and veterinary medicine, including third generation cephalosporins, other beta-lactams, aminoglycosides, tetracyclines and fluorochinolones, sulfonamides, chloramphenicol and others. Genotypic resistance screening via PCR included genes coding for ESBL-producing genes like $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{OXA} and the presence of common resistance genes like *tet*(A-C), *sul1*, *sul2*, *sul3*, *strA*, *strB*, *aadA1-like*, and *aac(3)-IV* as previously published by Kozak et al. [172] The phylogenetic background of all *E. coli* isolates was analysed using a PCR approach [20].

For selected isolates, the presence of plasmid-mediated chinolone resistance gene variant *aac(6´)-II-cr* and the *qnrA*, *qnrB*, and *qnrS* genes, as well as mutations in *gyrA* and *parC* genes, were determined by PCR and--if indicated--by sequence or restriction analysis [78, 79, 151, 173-178]. Most of the multi-resistant isolates were additionally processed via MIC testing (minimal inhibitory concentrations)[179, 180]. The production of ESBL was confirmed using the CLSI standard confirmatory test [1]. Depending on the scientific question, we performed a clonal analysis via PFGE (pulsed field gel electrophoresis) in most of the studies [181]. Additionally, an extensive virulence genotyping including more than 50 VAGs (virulence-associated genes) typical for ExPEC and InPEC was performed in some of the studies [16, 182]. Finally, selected isolates were analysed via MLST (Multilocus Sequence Typing) [6], plasmid profile analysis, southern blotting, inc/rep typing [183] and transconjugation experiments to test the transferability of ESBL plasmids [79]. If MLST data was available, *E. coli* phylogenetic groups were determined by bioinformatics analysis based on the concatenated sequences of the seven housekeeping genes (http://pritch.bsd.uchicago.edu/structure).

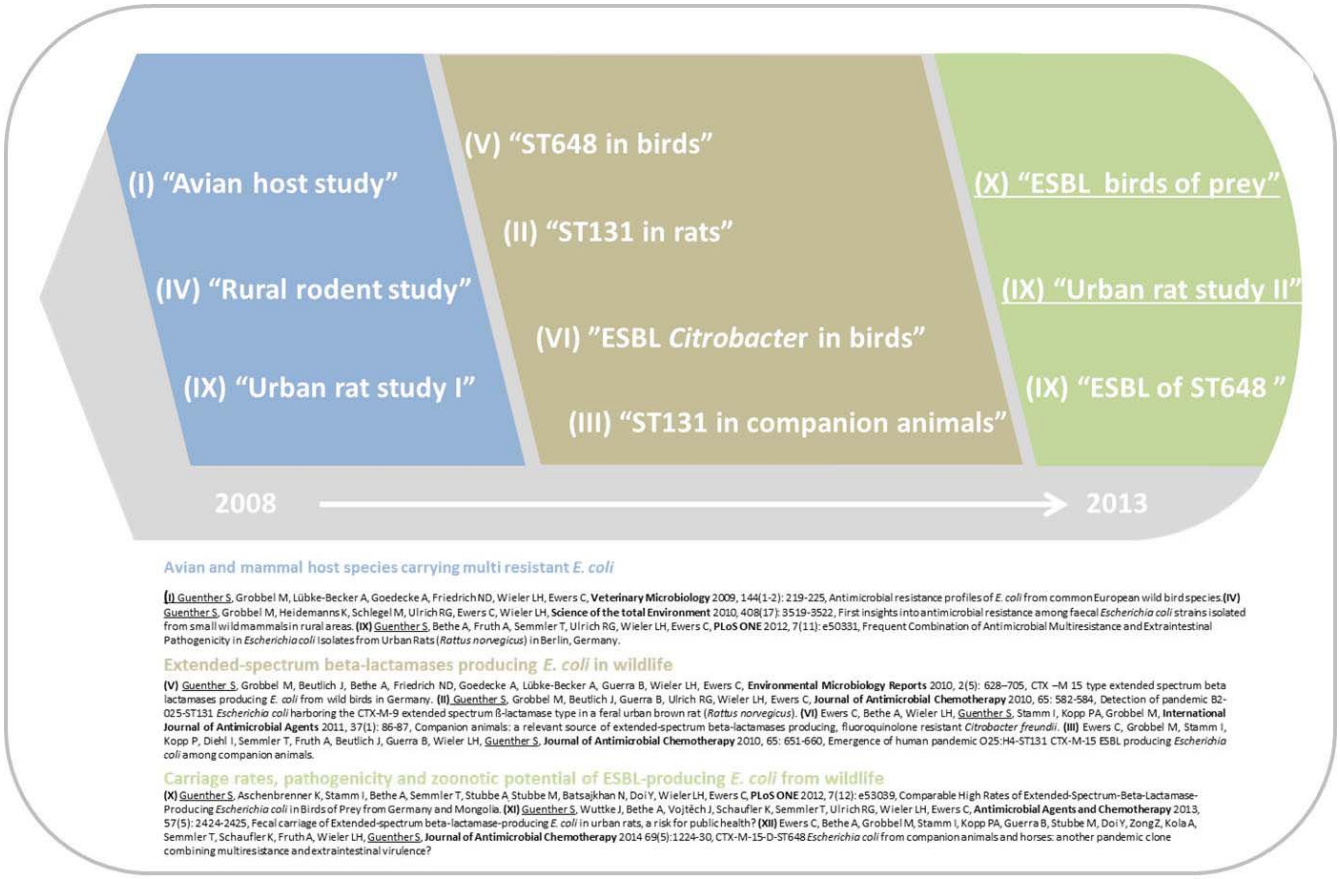


Fig. 9: Timeline and short title of the studies used for this habilitation thesis (excluding reviews), underlined: studies using selective media

2.1. Avian and mammalian host species carrying multi-resistant *E. coli*

2.1.1. Common occurrence of multi-resistant *E. coli* in avian wildlife (I)

In a study designed to assess the risk of the distribution of InPEC (intestinal) and ExPEC (extra-intestinal pathogenic *E. coli*) by waterfowl to environmental sources, Mallard ducks (*Anas platyhrynchos*) were also screened for the occurrence of antimicrobial resistant *E. coli*. Overall, 10% of all *E. coli* clones were resistant to at least one antimicrobial compound and one clone was multi-resistant [2] (IMT16085, 1.6% of all *E. coli* clones). This multi-resistant clone displayed high MIC values against amoxicillin, ampicillin, ticarcillin, sulfonamides, tetracyclines and streptomycin.

Although only one multi-resistant strain was found in this study, the proportion of isolates displaying antimicrobial resistance against one compound was noticeable, leading to the assumption that wildlife might carry *E. coli* isolates displaying antimicrobial resistance.

To gain detailed information on the possible role of wild avian host species of multi-resistant *E. coli* a broad spectrum of common European bird species was screened as a prolongation of the work on Mallard ducks [168]. In this pilot study fifty-five different European wild bird species including passerines, waterfowl and birds of prey were tested for antimicrobial resistant *E. coli* (refer to Table 1 of [I] and to Tab. 2 of this work for details of the species tested). Birds were sampled in two areas of Germany, one rural (Eichsfeld region, Northern Thuringia, approx. 100 inhabitants/km^2, predominantly villages) and one medium-sized urban agglomeration area (conurbation of Giessen, Middle Hesse, approx. 300 inhabitants/km^2).

Plating of overall 277 samples from 226 individual birds on non-selective medium resulted in a total 201 isolates presenting 187 *E. coli* PFGE clones which were further processed. Pre-selection of multi-resistant *E. coli* isolates resulted in an overall rate of 17.1% of the isolates showing phenotypic resistance to at least one antimicrobial substance. 8% of the isolates (n=15) were classified as multi-resistant (refer to Table 2 of [I] for results of MIC testing). The most abundant pattern observed was a combined resistance to amoxicillin, ampicillin, tetracycline, sulfamethoxazole and streptomycin.

Isolates with highest numbers of resistances were obtained from the following avian hosts: Buzzard (*Buteo buteo*, all from Hesse), Garden Warbler (*Sylvia borin*, Eichsfeld region), Grey Heron (*Ardea cinerea*, Hesse), Mute Swan (*Cygnus olor*, Hesse), Pigeons (*Columba livia,* both from Hesse) and a White-throated Dipper (*Cinclus cinclus*, Eichsfeld region, refer to Table 1 of [I] for the avian species with lower numbers of resistance and to Table 2 of [I] for detailed genotyping results). There was no significant difference in the isolation frequency of multi-resistant isolates between rural or urban areas (4.4% rural Eichsfeld region, 5.0%, urban area of Giessen). Phylogenetic typing of the *E. coli* isolates revealed that a large proportion of the multi-resistant isolates belonged to group B2 (46%). As we detected several birds of prey species as carriers of multi-resistant *E. coli,* it seemed thorough to include rodents and small mammals in our subsequent sampling schemes, as raptors largely feed on those animals.

2.1.2. Low rates of multi-resistant *E. coli* in different rodent and small mammal species from rural areas (IV)

Besides the screening of different rodent and small mammal host species the aim of this study was also to determine to what extent antimicrobial resistant *E. coli* isolates are prevalent in rural rodents in association with the livestock densities of the area they where trapped. Overall 1,443 animals, originating from different rodent monitoring programmes (Network "Rodent borne pathogens"[184]) in Germany were investigated. Sampling sites included agri- and silviculturally used farmland and natural preserve areas. The livestock index/ha ranged from 0.3 to 1.8 [185]. Geographical location of sampling sites with successful isolation of *E. coli* isolates and the livestock indices are shown in Fig. 10.

The common wild rodents and small mammals belonged to the family Muridae and different shrew species (order Soricomorpha or Eulipotyphla), and the detailed species list can be found in Tab. 2. *E. coli* were isolated from 13% of the frozen gut samples and from nine small mammal species, and all 188 isolates were assigned to individual clones by PFGE.

Pre-selection for resistant clones (method according to [**I**]) resulted in ten *E. coli* clones originating from three rodent species (Wood mouse [*A. sylvaticus*], Yellow-necked mouse [*A. flavicollis*] and Bank vole [*Myodes glareolus*]), with phenotypic resistance against at least one antimicrobial compound. Overall, four clones (2.2%) were multi-resistant and most of them (n=3) originated from Wood mice.

The overall level of antimicrobial resistance to aminoglycosides, tetracyclins, aminopenicillins and sulfonamides observed among *E. coli* isolates was low, and resistance to cephalosporins, chloramphenicol and fluorochinolones were not observed at all (refer to Tab. 1 of [IV] for details of the strain/resistance patterns). Nevertheless, we observed a statistically significant tendency that *E. coli* from wild rodents with antimicrobial resistance against more than one antimicrobial substance where more isolated from areas with livestock breeding than from woodland areas. By taking into account the overall German livestock unit (1.1 Livestock Units/hectare), we divided the 188 clones according to their sampling sites into two subgroups (Fig. 10): A (n=107), woodland environments with very low rates of livestock (<0.6 LU/hectare), and B (n=81), intensively used farmland with high rates of livestock (>1.0 LU/hectare). A significant correlation between the type of area and the occurrence of isolates resistant to more than one antimicrobial was detectable (p<0.05), as most of these clones were sampled in subgroup B areas (farmland with the highest density of livestock breeding in Germany (>1.8 LU/hectare) (refer to [IV] for details).

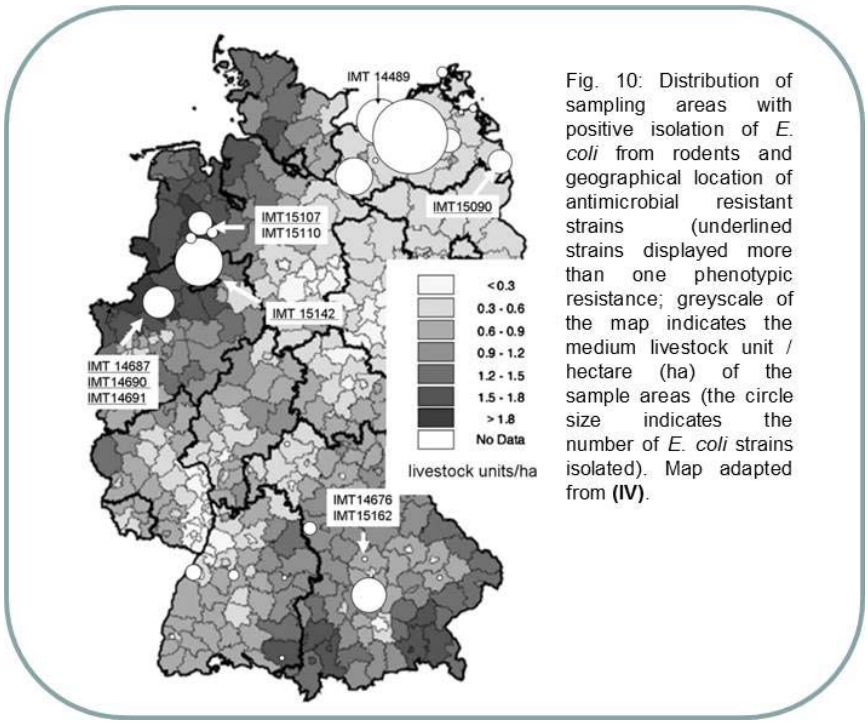


Fig. 10: Distribution of sampling areas with positive isolation of *E. coli* from rodents and geographical location of antimicrobial resistant strains (underlined strains displayed more than one phenotypic resistance; greyscale of the map indicates the medium livestock unit / hectare (ha) of the sample areas (the circle size indicates the number of *E. coli* strains isolated). Map adapted from **(IV)**.

As all of the *E. coli* clones exhibiting multi-resistance originated from rodents captured in close proximity to intensively used farmland or livestock breeding, we wanted to find evidence of whether synanthropic behaviour resulting in the usage of human infrastructure by the animals might be a key issue for antimicrobial resistance in wildlife. I therefore continued my work by screening a highly anthropophilic urban species, the Brown rat.

2.1.3. High rates of multi-resistant *E. coli* in urban Brown rats (IX)

Brown rats (*Rattus norvegicus*) have presented a global public health concern for centuries, as they are considered a reservoir and vector of zoonotic pathogens like the plague. The intention of my first rat study was to analyse whether rats are a potential source of putatively zoonotic and antimicrobial resistant *E. coli.* For that, in 2008-2009 faecal samples of 87 Brown rats from 53 different sampling locations in Berlin (Germany) were screened by plating on non-selective medium.

Overall, we observed a high detection rate of *E. coli* from rats (77.0%). From a total of 211 non-duplicate clones fifty-five (26%) exhibited a resistant phenotype against at least one antimicrobial class and overall thirty-two (13.6%) of the *E. coli* were multi-resistant [2] (refer to Tab. 1 of [**IX**] for details). The most abundant pattern observed was combined resistance against ampicillin, tetracycline and fluorochinolones. Of these multi-resistant isolates, two showed a positive confirmatory test for the production of ESBLs (IMT19205 and IMT20717). Screening for antimicrobial resistance determinants almost always reflected the phenotypic resistance situation. Most or all isolates harboured $bla_{\text{TEM-1-like}}$ (87.5%), *sul1/sul2* (75%) and *strA/B* genes (100%), whereas other non-beta-lactam resistance genes, such as *aadA* (34.4%), *tet*(A-D) (25%), *aac(3)IV* (3.1 %), *aac(6`)-Ib-cr* (6.3%), and *qnrB1* (3.1%) were present in lower frequencies (refer to Tab.1 of [**IX**] for detailed genotyping data).

MLST led to the detection of a total of 24 different STs among the 32 multi-resistant isolates, whereof seven were assigned to ancestral group B2, four to group D, three to group B1, two to group A, eleven to hybrid group ABD and five to hybrid group AxB1 [6] (refer to Tab.1 of [**IX**] for details). In conclusion, more than one third (34.5%) of multi-resistant isolates were allocated to the ExPEC-linked phylogenetic groups B2 and D. During the sampling for two of my studies (**I, IX***), E. coli* isolates with an ESBL-phenotype were detected in wild birds and wild rats.

These isolates were characterized in detail and compared to ESBL-producing isolates from companion animals and humans and the results are given in the next section of this work. Overall, this study also showed that the detection rates of multi-resistant *E. coli* from the urban rodents were tenfold higher than in rodents from rural areas underlining the influence of synanthropism **(IV).**

2.2. Extended-spectrum beta-lactamases producing *E. coli* in wildlife

At this point in time (2008) the first ESBL-producing *E. coli* from wildlife had only been described two years before [147], and very little was known about the occurrence of ESBL outside clinical settings.

2.2.1. Detection of ESBL-producing *E. coli* in wild birds from Germany (V)

Four avian *E. coli* isolates from our original study in Germany (**I**) showed a positive phenotypic confirmatory test for ESBL-production. Avian hosts, which carried these ESBL-producing *E. coli,* originated from different areas of Hesse (Germany) and were isolated in 2006. The four isolates were recovered from four individual birds (2.3% of 172 birds sampled), namely a Rock Pigeon (*Columba livia)*, a Greater White-fronted Goose, (*Anser albifrons*) and two Eurasian Blackbirds (*Turdus merula*). All isolates revealed identical MICs for non-beta-lactam antibiotics, including resistance against gentamicin, different fluorochinolones, sulfa-methoxazol/trimethoprim and tetracycline (refer to Tab. 1 of **[V]** for detailed MIC data). The beta-lactamase genes detected in these isolates were $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$. The $bla_{\text{CTX-M-15}}$ gene was located on a transferable multi-replicon plasmid (>100kb) positive for the FII, FIA and FIB replicon-types. The CTX-M-15-producing *E. coli* also harboured genes conferring resistance against tetra-cycline *tet(A), tet(B)* and aminoglycosides (*aadA2*, *strA*, *strB*). Phylogenetic grouping assigned the CTX-M-15-producing *E. coli* to group D and ST648. PFGE resulted in identical macro-restriction patterns (Fig. 11), suggesting a clonal relationship of the four *E. coli* isolates, although they were recovered from individuals from different avian species.

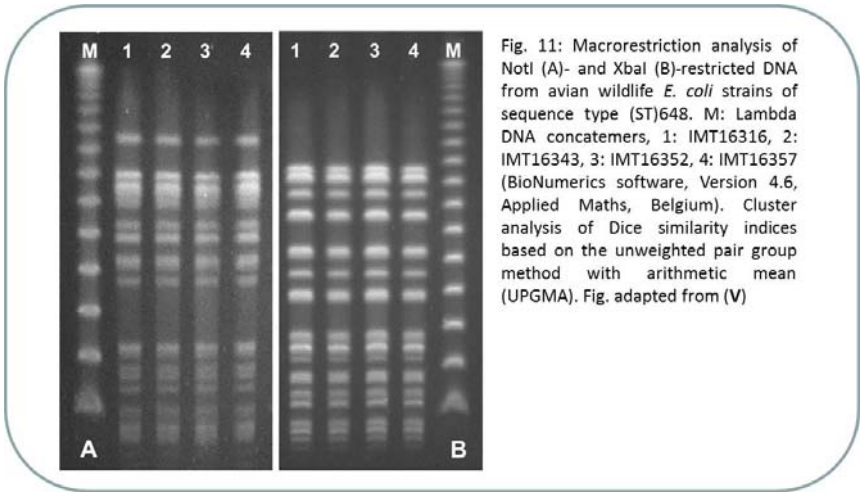
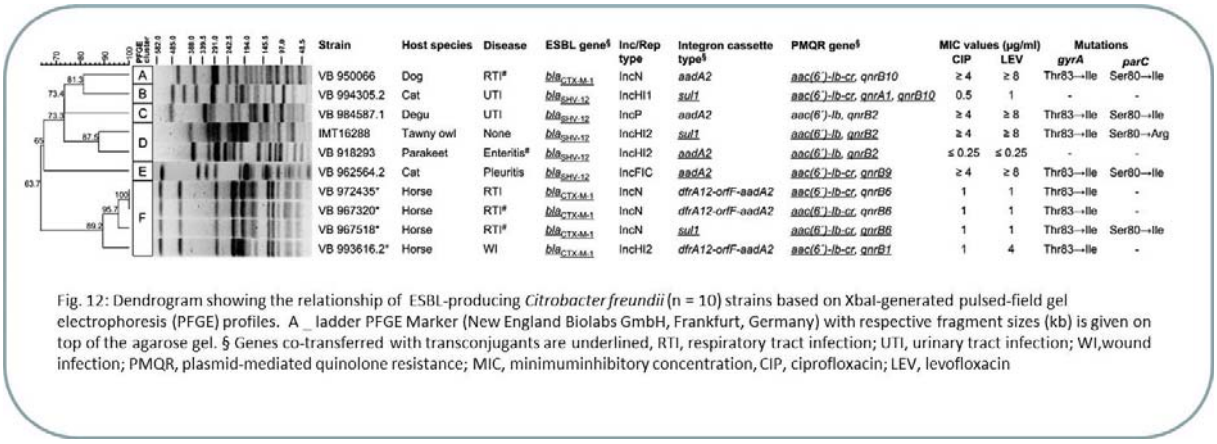


Fig. 11: Macrorestriction analysis of NotI (A)- and XbaI (B)-restricted DNA from avian wildlife *E. coli* strains of sequence type (ST)648. M: Lambda DNA concatemers, 1: IMT16316, 2: IMT16343, 3: IMT16352, 4: IMT16357 (BioNumerics software, Version 4.6, Applied Maths, Belgium). Cluster analysis of Dice similarity indices based on the unweighted pair group method with arithmetic mean (UPGMA). Fig. adapted from (V)

2.2.2. ESBL-producing *Citrobacter freundii* in wild birds (VI)

Besides ESBL-producing *E. coli*, other multi-resistant Enterobacteriaceae including *Klebsiella pneumoniae* subsp*. pneumoniae* or *Citrobacter freundii* have also become a serious problem in the treatment of infectious diseases.

We therefore explored the occurrence of ESBL-producing Enterobacteriaceae other than *E. coli* in wild animals as well and compared them to isolates from companion animals collected in a veterinary diagnostic lab covering Central Europe. The two avian Enterobacteriaceae isolates originated from the wild bird study in Germany (I) and showed phenotypic resistance against cefotaxime. One of these isolates turned out to be *Enterobacter cloacae* with presumably intrinsic AmpC-production. For one *Citrobacter freundii* isolate (IMT16288), ESBL-production was confirmed. This strain originated from a Tawny owl (*Strix aluco*). It displayed a multi-resistant phenotype with high MIC values, indicating resistance against fluorochinolones, gentamicin, tobramycin, trimethoprim/sulfonamide and tetracyclines. *bla*$_{SHV-12}$ was the ESBL-encoding gene and the other resistance genes detected confirmed the phenotypic results. Macro-restriction analysis of the strain and comparison to domestic animal isolates revealed a close relatedness to one strain originating from a domestic parakeet (Fig. 12).



	Strain	Host species	Disease	ESBL gene§	Inc/Rep type	Integron cassette type§	PMQR gene§	MIC values (µg/ml) CIP	MIC values (µg/ml) LEV	Mutations *gyrA*	Mutations *parC*
A	VB 950066	Dog	RTI#	$bla_{CTX-M-1}$	IncN	*aadA2*	*aac(6')-Ib-cr, qnrB10*	≥ 4	≥ 8	Thr83→Ile	Ser80→Ile
B	VB 994305.2	Cat	UTI	bla_{SHV-12}	IncHI1	*sul1*	*aac(6')-Ib-cr, qnrA1, qnrB10*	0.5	1	-	-
C	VB 984587.1	Degu	UTI	bla_{SHV-12}	IncP	*aadA2*	*aac(6')-Ib, qnrB2*	≥ 4	≥ 8	Thr83→Ile	Ser80→Ile
D	IMT16288	Tawny owl	None	bla_{SHV-12}	IncHI2	*sul1*	*aac(6')-Ib, qnrB2*	≥ 4	≥ 8	Thr83→Ile	Ser80→Arg
	VB 918293	Parakeet	Enteritis#	bla_{SHV-12}	IncHI2	*aadA2*	*aac(6')-Ib, qnrB2*	≤ 0.25	≤ 0.25	-	-
E	VB 962564.2	Cat	Pleuritis	bla_{SHV-12}	IncFIC	*aadA2*	*aac(6')-Ib-cr, qnrB9*	≥ 4	≥ 8	Thr83→Ile	Ser80→Ile
F	VB 972435*	Horse	RTI	$bla_{CTX-M-1}$	IncN	*dfrA12-orfF-aadA2*	*aac(6')-Ib-cr, qnrB6*	1	1	Thr83→Ile	-
	VB 967320*	Horse	RTI#	$bla_{CTX-M-1}$	IncN	*dfrA12-orfF-aadA2*	*aac(6')-Ib-cr, qnrB6*	1	1	Thr83→Ile	-
	VB 967518*	Horse	RTI#	$bla_{CTX-M-1}$	IncN	*sul1*	*aac(6')-Ib-cr, qnrB6*	1	1	Thr83→Ile	Ser80→Ile
	VB 993616.2*	Horse	WI	$bla_{CTX-M-1}$	IncHI2	*dfrA12-orfF-aadA2*	*aac(6')-Ib-cr, qnrB1*	1	4	Thr83→Ile	-

Fig. 12: Dendrogram showing the relationship of ESBL-producing *Citrobacter freundii* (n = 10) strains based on XbaI-generated pulsed-field gel electrophoresis (PFGE) profiles. A _ ladder PFGE Marker (New England Biolabs GmbH, Frankfurt, Germany) with respective fragment sizes (kb) is given on top of the agarose gel. § Genes co-transferred with transconjugants are underlined, RTI, respiratory tract infection; UTI, urinary tract infection; WI,wound infection; PMQR, plasmid-mediated quinolone resistance; MIC, minimuminhibitory concentration, CIP, ciprofloxacin; LEV, levofloxacin

2.2.3. Occurrence of ESBL-producing *E. coli* in urban Brown rats (II, IX)

Two *E. coli* isolates (IMT19205 and IMT20717) sampled during my study on multi-resistant *E. coli* from urban rats **(IX)** showed a positive confirmatory test for ESBL-production. For one of the isolates (IMT19205), MLST and molecular determination of the rfb25b locus by PCR [186] revealed sequence type ST131 and O antigen subtype O25b. MIC testing showed resistance against several 1-3 [gen.] cephalosporins, aminopenicillins, tetracyclins, aminoglycosides and fluorochinolones.

Genotyping of resistance genes revealed beta-lactamases types $bla_{\text{CTX-M-9}}$ and $bla_{\text{TEM-1}}$ as well as non-beta-lactamase resistance genes like *tet(A), sul2, strA, aac(3)IV* and *aac(6´)-Ib-cr*.

The genes were located on a self-transferable plasmid (>100kb) of the FIA/FIB replicon type. Additionally, PFGE revealed a close genetic similarity to a control strain of the O25b-ST131 *E. coli* human pandemic clonal group, as depicted in Fig. 13.

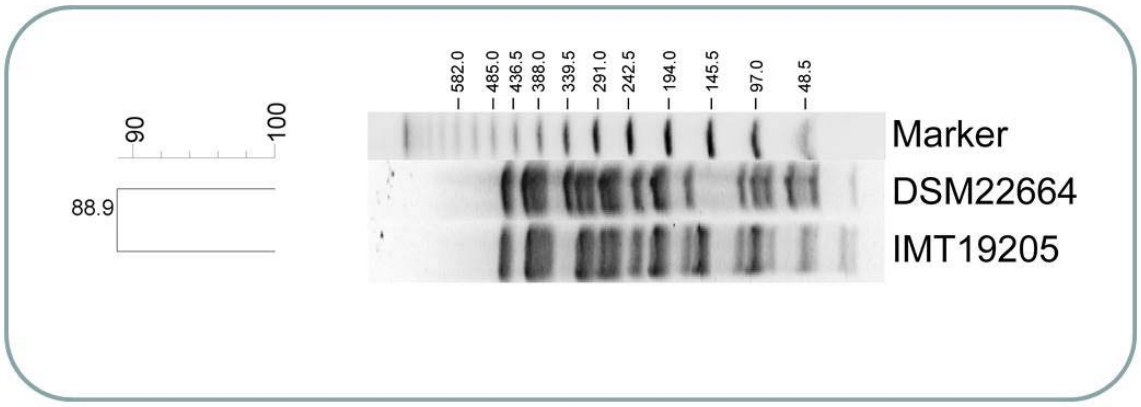
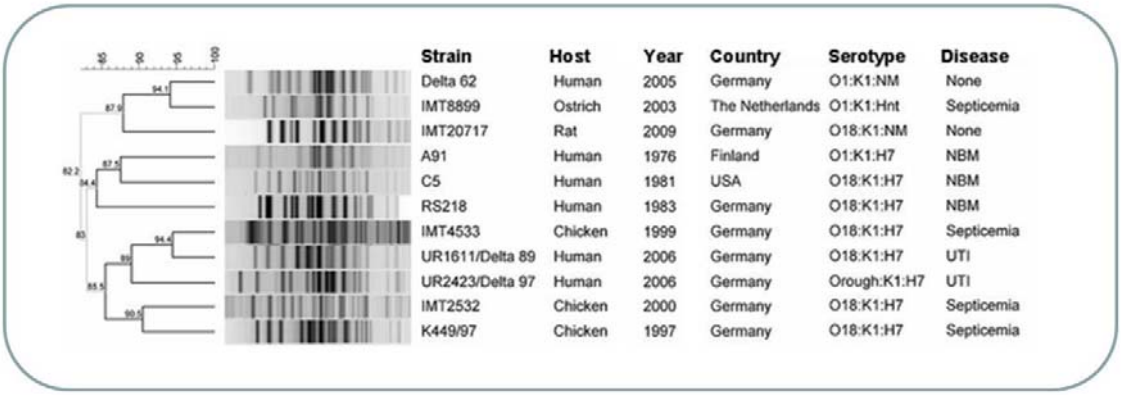


Fig. 13: Dendrogram (BioNumerics®, Version 4.6, Applied Maths, Belgium) of CHEF-PFGE-with XbaI-restricted genomic DNA of CTX-M-9-type ESBL *E. coli* (IMT19205) strain of sequence type ST131 and human control strain DSM 22664 (RL102/05; O25b-CTX-M-15-ST131)

MLST analysis of the second ESBL-producing *E. coli* isolate from a rat (IMT20717) revealed a sequence type clearly associated with extra-intestinal pathogenicity in *E. coli*: ST95 [187, 188].

Phenotypic antimicrobial resistance to several beta-lactams and non-beta-lactams was detected for this clone as well. Apart from a $bla_{\text{CTX-M-9}}$ gene, this strain also harboured resistance genes $bla_{\text{TEM-1}}$, *sul2*, *strA*, *strB, aac(6´)-Ib-cr* and *aadA.* All these genes, except for *sul2*, were located on a self-transferable, approximately 50 kb plasmid of the N/FIC replicon type. Serotyping characterized strain IMT20717 as O18:NM:K1.

Clonal analysis revealed a high genetic similarity (Dice similarity $\geq$ 82.2%) between the rat B2-ST95-O18:NM:K1-CTX-M-9 isolate and clinical ST95 isolates of different ExPEC pathovars, as well as an additional faecal isolate from the gut of a healthy human (Fig. 14, p. 42).



Strain	Host	Year	Country	Serotype	Disease
Delta 62	Human	2005	Germany	O1:K1:NM	None
IMT8899	Ostrich	2003	The Netherlands	O1:K1:Hnt	Septicemia
IMT20717	Rat	2009	Germany	O18:K1:NM	None
A91	Human	1976	Finland	O1:K1:H7	NBM
C5	Human	1981	USA	O18:K1:H7	NBM
RS218	Human	1983	Germany	O18:K1:H7	NBM
IMT4533	Chicken	1999	Germany	O18:K1:H7	Septicemia
UR1611/Delta 89	Human	2006	Germany	O18:K1:H7	UTI
UR2423/Delta 97	Human	2006	Germany	Orough:K1:H7	UTI
IMT2532	Chicken	2000	Germany	O18:K1:H7	Septicemia
K449/97	Chicken	1997	Germany	O18:K1:H7	Septicemia

Fig. 14: Dendrogram of ST95-ESBL rat strain IMT20717 with *E. coli* ST95-K1 strains. The clonal relationship shown is based on *XbaI*-generated PFGE profiles. NM = non motile (H antigen negative or not expressed); NBM = newborn meningitis; UTI = urinary tract infection, optimization 1.0%, position tolerance 1.5%.

2.3. Carriage rates, pathogenicity and zoonotic potential of ESBL-producing *E. coli* from wildlife

During the first part of my work, the mere occurrence of zoonotic ESBL-producing *E. coli* in different wildlife species was proven. Several new implications arose from these findings, as, besides basic questions concerning colonization of these strains , the pathogenic potential as well as the carriage rate of ESBL-producing *E. coli* and possible modes of transmission are of utmost importance. My subsequent work therefore focused on some of these topics, including carriage rates and possible transmission routes. To gain information on the carriage rate of ESBL-producing isolates in wild avian hosts in areas influenced differently by humans, a comparative study was performed **(X).** In parallel to that, the screening of urban rats was continued using selective screening for ESBL-producing *E. coli* (**XI**). Furthermore, we analysed the pathogenic potential of multi-resistant *E. coli* isolates from rats in an animal experiment **(IX**). Finally, the comprehensive analysis of our data from wildlife and companion animals led to the detection of a pandemic clonal lineage of ST648 in different hosts underlining the zoonotic nature of ESBL-producing *E. coli* **(XII)**.

2.3.1. Comparable rates of ESBL-producing *E. coli* in birds of prey from Mongolia and Germany (X)

In 2010 sixteen avian species were comparatively screened for the occurrence of ESBL-producing *E. coli* in Mongolia and Germany. Most of the avian hosts sampled were birds of prey. Some of the host species tested can be found in both areas, but the populations are not connected via bird migration. Sampling was carried out using cloacal swabs in Central Germany, Saxony-Anhalt, in the Northern region of the Harz-mountains (human density: 116 n/km^2, livestock densities: cattle/swine 50–100 n/km^2, small ruminants 10 n/km^2, poultry 1.000–2.500 n/km^2) and at several sampling spots in the South-Mongolian semi-desert and in West Mongolia (for detailed geographic origin refer to Tab. 1 in [**X**], human density: 1–2 n/km^2, livestock densities: swine <1 n/km^2, cattle 1–5 n/km^2, small ruminants 5–10 n/km^2, poultry <10 n/km^2). Cloacal swabs were streaked out on selective CHROMagar orientation containing 4 µg/ml cefotaxime and the same plates without the antimicrobial compound. We isolated comparable rates of *E. coli* from birds sampled in both areas, as 38% of the German and 41% of the Mongolian birds carried *E. coli*.

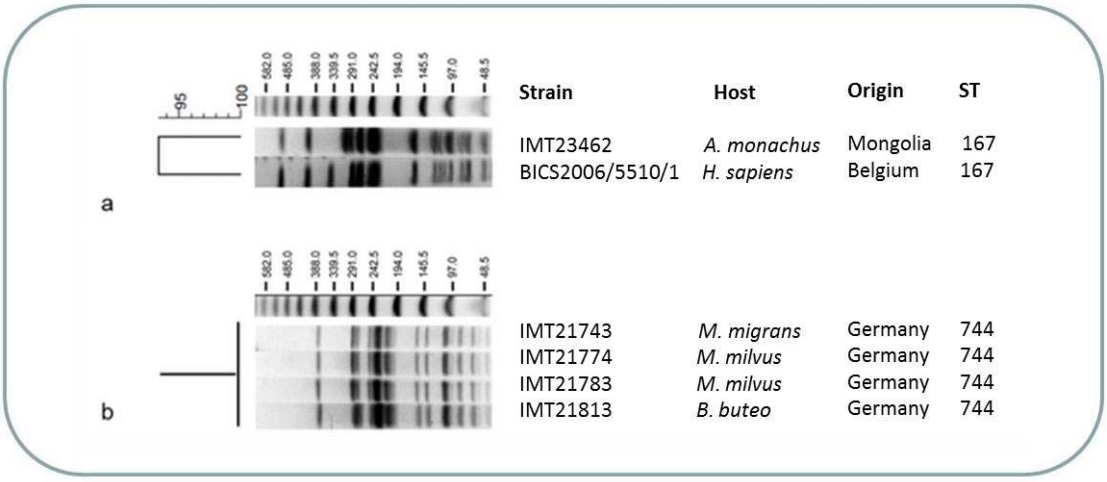


Fig. 15: (a) dendrogram of *Xba*I PFGE profiles of avian and human ESBL-producing *E. coli* of ST167, (b) four avian ST744 ESBL-producing *E. coli* (Bionumerics 6.6 ,Applied Maths, Belgium) ST = sequence type; *A.* = *Aegypius; B.* = *Buteo*; *H.* = *Homo*; *M.* = *Milvus,* A size marker (Lambda Ladder PFGE Marker; New England Biolabs GmbH, Frankfurt a. M., Germany)

We confirmed ESBL-production in 13.8% (n = 9) of the German and in 10.8% (n = 4) of the Mongolian *E. coli* isolates. All ESBL-producing *E. coli* from this study originated from different individual raptors in different nests, thus precluding a possible bias caused by inter-sibling transfer in the nest.

Including all birds of the study--even those, which did not carry *E. coli*--5.2% of the wild birds from Germany and 4.5% of those from Mongolia carried ESBL-producing *E. coli*. Genotypic characterization of the isolates revealed $bla_{\text{CTX-M-1}}$ predominating among German (100%) and $bla_{\text{CTX-M-group-9}}$ amongst Mongolian isolates (80%).

The phenotypic resistance patterns of these isolates confirmed the genotypic results (refer to Tab. 2 of **[X]** for detailed genotyping data), and all ESBL-producers were multi-resistant (refer to Tab. 2 of **[X]** for detailed genotypic and phenotypic data). Overall, ten different STs were detected among the avian ESBL-producing *E. coli*, including ST12 (ancestral group B2), ST847 (B1), ST167 (A), and ST117 (ABD).

Interestingly, ST167 belongs to STs that have been associated with the global carriage of ESBL-producing *E. coli* in humans [47]. In one of these previously published studies on the epidemiology of ESBL-producing Enterobacteriaceae in Belgian hospitals [189], an ESBL-producing ST167 *E. coli* isolate (BICS2006/5510/1) was detected in a clinical sample from a 67-year-old patient with urinary tract infection. Indeed, a comparative macro-restriction analysis of this strain with the Mongolian ST167 Black vulture (*A. monachus*) ESBL-producing *E. coli* isolate IMT23462 confirmed the clonal relatedness of both isolates (dice similarity index >90%) (Fig. 15a, p. 43). Interestingly, four of the isolates collected from wild birds in Germany were assigned to ST744 and were subsequently found to belong to a single and identical PFGE clone (Fig. 15b, p. 43), although they originated from individuals (belonging to three avian species) sampled at different locations within an area of 30 square kilometres.

2.3.2. Paradigmatic combination of virulence and multi-resistance in an ESBL-producing isolate from a rat (IX)

During my studies we often obtained ESBL-producing isolates, which were not only multi-resistant but could be either assigned to phylogenetic backgrounds associated with ExPEC and/or harboured virulence associated genes typical for this pathotype. In contrast to InPEC, the definition of ExPEC based on the occurrence of certain pathogenicity markers is not fully solved up to date [190, 191].

Thus, the definition of an ExPEC can currently only be made with animal experiments to prove its pathogenic potential, as the possession of virulence genes alone is only a prerequisite of pathogenicity. As an example, in the first rat study in 2010 (I**X**), we frequently detected ExPEC-related genes in rodent isolates. Overall, 17.2% of all multi-resistant rat isolates harboured at least twenty ExPEC-VAGs.

The presence of typical ExPEC-related adhesins, such as the heat-resistant agglutinin *hrA,* iron-regulated hemagglutinin *iha or* P-fimbriae (*pap* operon genes), gave a hint towards the affiliation of a number of rat isolates to the pathotype ExPEC. Iron acquisition genes, such as *chuA*, *fyuA*, *iroN*, *irp2*, *iucD*, *iutA*, *sitA*, and $sitD_{episomal}$, which are known to confer fitness advantages and also invasive properties towards *E. coli,* were also frequently detected.

Additionally, we found protectin genes like *iss* and invasion-associated K1-capsule encoding gene *neuC*, as well as the plasmid-located transfer gene *traT* and outer membrane genes like *ompT*. Toxin genes, such as the cytonecrotizing factor encoding *cnf*, secreted autotransporter toxin *sat*, vacuolating autotransporter toxin *vat*, and haemolysin operon genes *hlyA* and *hlyC* were also found (refer to Fig. 3 of **[IX]** for detailed virulence genotyping data). Nevertheless, to prove the pathogenic potential of these hybrid strains which combine antimicrobial resistance with extraintestinal virulence potential, we analysed one B2 ST95-O18:NM:K1-CTX-M-9 strain (IMT20717) as a paradigm for resistance, possession of virulence associated genes and a phylogenetic background associated with ExPEC experimentally in an established *in vivo* chicken infection model [192].

Although the tested strain IMT20717 revealed a lower bacterial recovery rate from chicken organs than the highly virulent positive control strain (RS218, a human clinical NMEC type strain), the strain could be isolated from all internal organs in significantly higher numbers than the negative control (IMT12226, low pathogenic avian control strain) (Fig. 16, p. 46). Particularly the re-isolation of IMT20717 from the brain is of high indicative value for its invasive potential, as it suggests that the strain is able to penetrate the blood-brain barrier.

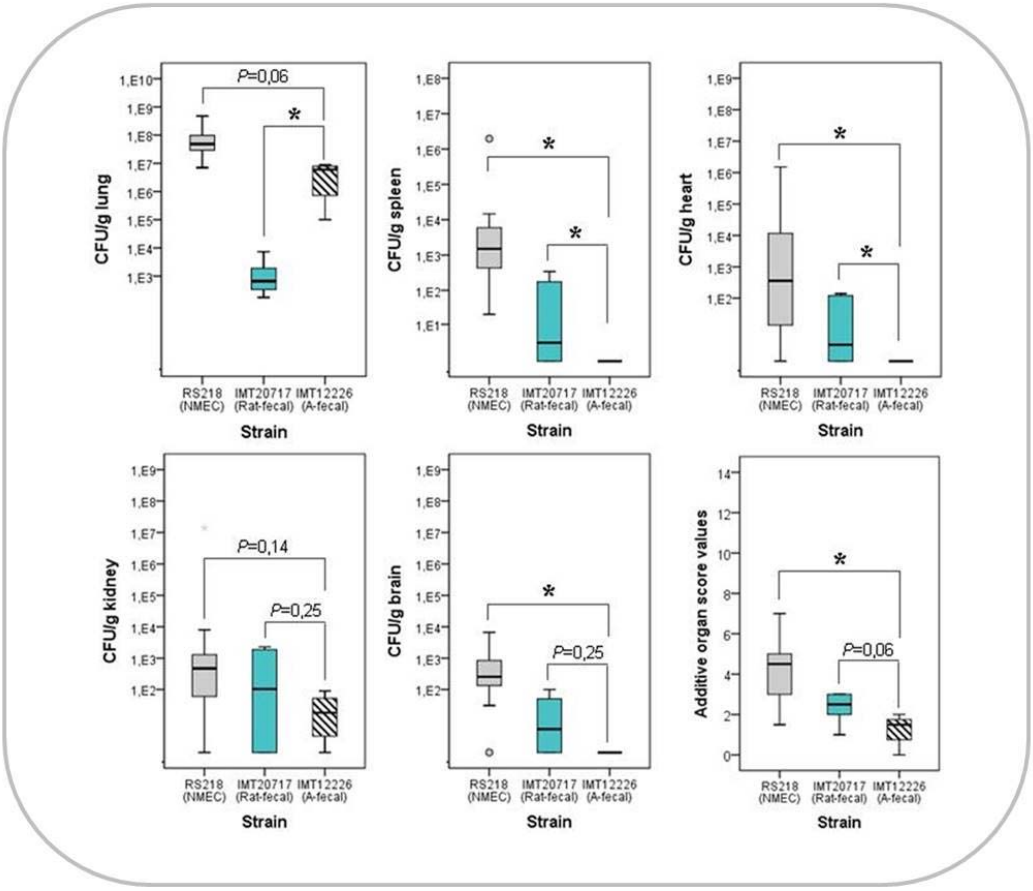


Fig. 16: Results of a ESBL-producing *E. coli* of ST95 from a rat (*R. norvegicus*, IMT20717) in the chicken infection model**.** Ability of B2-ST95-O18:NM:K1-CTX-M-9 (IMT20717) to colonize the lungs, disseminate into internal organs and penetrate the blood brain barrier 24 h post intra-tracheal infection (10^9 CFU) of a group of six 5-weeks old SPF White Leghorn chickens. Non-ESBL-producing NMEC strain RS218 (B2-ST95-O18:H7:K1) and avian fecal strain IMT12226 (ST1165-O77:H18), known invasive and low pathogenic strains, were used as controls.

2.3.3. High carriage rates of ESBL-producing *E. coli* in urban rats (XI)

According to my previous studies (**II, IX**), ESBL-producing *E. coli* are apparently present in faeces of urban Brown rats (*R. norvegicus*) from Europe [117, 165]. In 2010, we screened a total of 56 Brown rats for ESBL-producing *E. coli* by plating faecal content using selective chrome-agar orientation plates (4µg/ml cefotaxime) to determine carriage rates of ESBL-producing *E. coli*. Rats were either trapped during pest control procedures in buildings and public areas like parks and streets (n=47) or in sewer tunnels close to the wastewater discharge of a university hospital (n=9). Overall, 16% of the rats examined carried an ESBL-producing *E. coli* and rats from the sewer tunnels twice as often (33%). The most prevalent ESBL-gene detected among the rat isolates was $bla_{\text{CTX-M-1}}$ (87.5%). All ESBL-producing *E. coli* harboured transferable large resistance plasmids of >100kB belonging to inc/rep type FIA or FIB.

Most of the isolates showed combined resistance to other antimicrobial classes including fluorochinolones, tetracyclines and aminoglycosides (refer to Fig. 17 for detailed genotyping data). Sequence types found included ST10 ST410, or ST90.

Interestingly, the ST90 ESBL-producing *E. coli* isolates could be traced via PFGE in three different animals over a period of two months presenting a single clone (Fig. 17, grey boxed). This clone initially appeared in two animals from the same sampling spot in the sewage system within two weeks. Six weeks later, it was recovered from a third animal in a nearby flat.

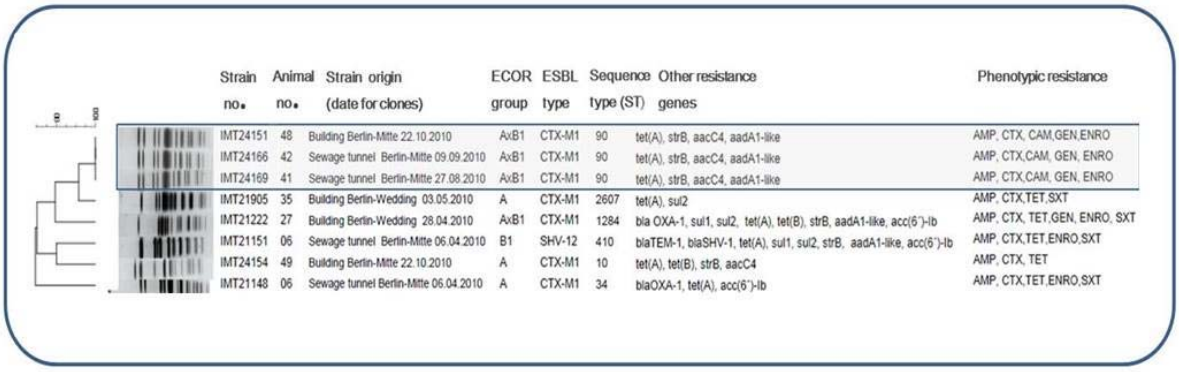


Fig. 17: Genotypic and phenotypic characteristics of ESBL-producing *E. coli* isolates from urban rats based on a dendrogram using *Xba*I-generated PFGE profiles Marked in grey: based on a similarity index of 100 % three *E. coli* strains present a clone originating from three different animals, two different locations and three different time points. Abbreviations: no.= number

2.3.4. Intercontinental dissemination of CTX-M-producing extra-intestinal pathogenic *Escherichia coli* D-ST648 among domestic animals, wild birds and humans (XII)

Throughout two of my studies we detected ESBL-producing *E. coli* of phylogroup D and sequence type (ST) 648 **(V, X)** in wild birds. A survey of the current literature indicated that ESBL-producing *E. coli* of this ST had also been isolated from humans and livestock at this time [47]. A comparison of ST648 isolates from different backgrounds seemed therefore interesting, in order to elucidate if, besides ST131 other "ESBL-associated ST" exist. ESBL-producing *E. coli* isolates, consecutively collected from veterinary clinical samples (mostly dogs, cats and horses) were therefore screened for isolates belonging ST648. Additionally, human isolates and all wildlife ST648 isolates previously isolated in my studies were included for comparative reasons.

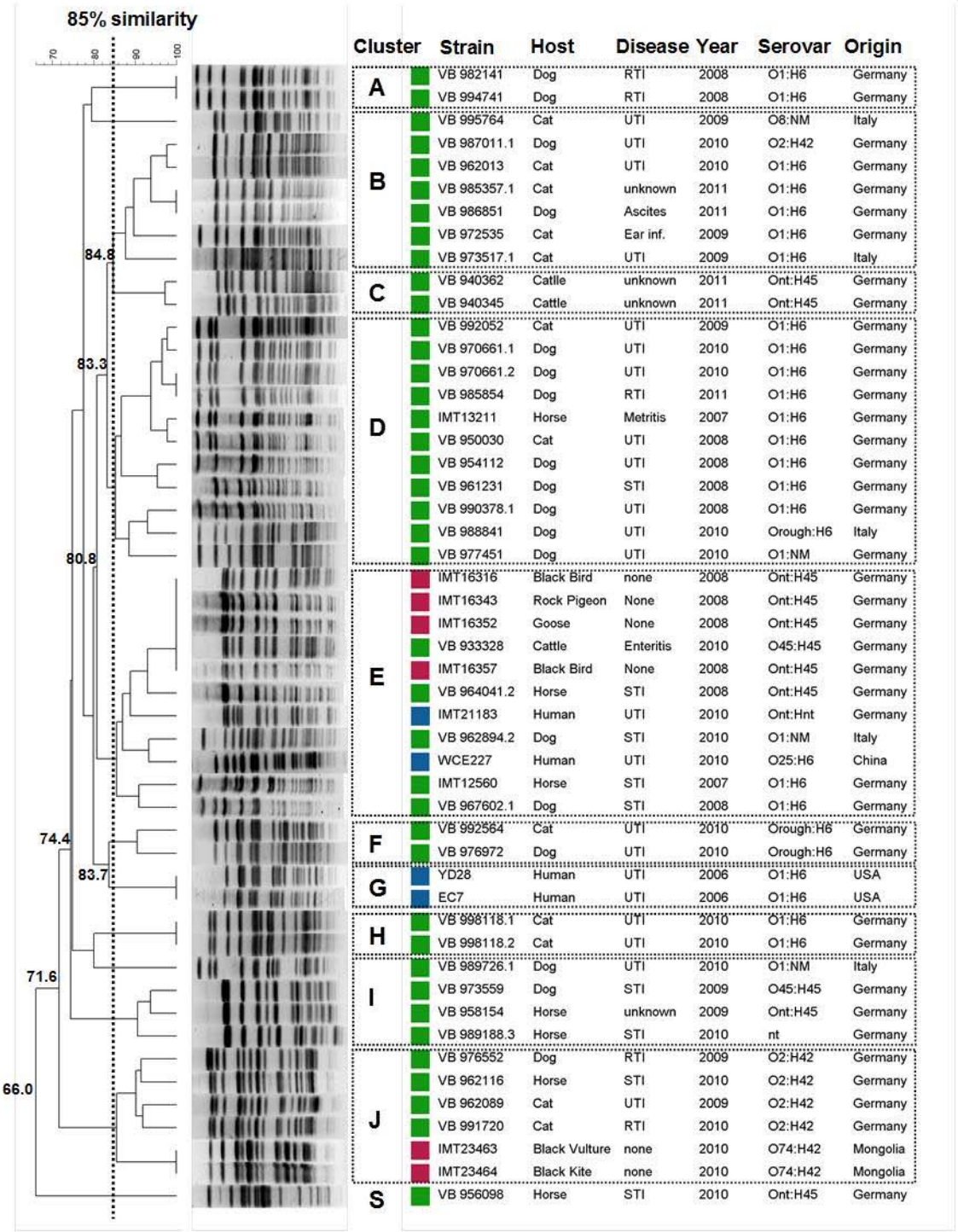
Two hundred of the 1.152 clinical ESBL-producing *E. coli* investigated (17.4%) were determined as EcoR group D isolates and 40 could be confirmed as ST648 after MLST (overall

3.5% of the entire ESBL-producing *E. coli*). Determination of resistance phenotypes and genotypes revealed that the 40 D-ST648 ESBL-producing *E. coli* isolates from domestic animals revealed high antimicrobial resistance levels (refer to Table II of **[XII]** for detailed genotyping data and to supplemental Table 1 of **[XII]** for MIC data).

The $bla_{CTX\text{-}M}$ and other non-beta-lactam resistance genes of the ST648 isolates were located on large plasmids with different replicon types (refer to Table II of **[XII]**). A comparative clonal analysis for 47 CTX-M-producing D-ST648 isolates was performed, including four human and six wild avian isolates. Overall, we found ten PFGE clusters of clonally related groups (Fig. 18).

In some of them, isolates from wildlife, domestic animals and humans clustered together. As an example, the similarity value for isolates within Cluster J was >85% (Fig. 18, p. 49), and this cluster comprised the two avian isolates from Mongolia [193] and isolates from companion animals with different infections. Cluster E displayed a similarity of >91% and involved several isolates from wound infections in horses and dogs (IMT17887, IMT18984, VB962894.2, VB967602.1), enteritis in cattle (VB933328) and four previously described isolates from wild avian faeces (IMT16316, 16343, 16352, 16357)[166]. Additionally, two were from human isolates, namely from a German patient with urinary tract infection (IMT21183) and the other one from a Chinese patient with the same disease (WCE227). ST648-ESBL-producing *E. coli* revealed an inhomogeneous virulence gene profile. Among the VAGs tested, the isolates contained between 6 and 24 genes each (refer to supplementary Fig. 1 of **[XII]**).

Several VAGs were present frequently in all isolates tested, including adhesin genes, iron uptake related genes *chuA*, *feoB,* and*,* serum resistance related genes *ompA*. Seventeen isolates could be identified as ExPEC, as they possessed two or more of these virulence genes (*papC*, *iutA*, *kpsMTII*, *sfa/foc*, *afa/dra*) necessary for fulfilling previously defined ExPEC criteria [194]. Summing up this data, ESBL-producing D-ST648 isolates with a certain pathogenic potential from wildlife and veterinary or human clinical background are clonally related to each other.



Cluster	Strain	Host	Disease	Year	Serovar	Origin
A	VB 982141	Dog	RTI	2008	O1:H6	Germany
	VB 994741	Dog	RTI	2008	O1:H6	Germany
B	VB 995764	Cat	UTI	2009	O8:NM	Italy
	VB 987011.1	Dog	UTI	2010	O2:H42	Germany
	VB 962013	Cat	UTI	2010	O1:H6	Germany
	VB 985357.1	Cat	unknown	2011	O1:H6	Germany
	VB 986851	Dog	Ascites	2011	O1:H6	Germany
	VB 972535	Cat	Ear inf.	2009	O1:H6	Germany
	VB 973517.1	Cat	UTI	2009	O1:H6	Italy
C	VB 940362	Cattle	unknown	2011	Ont:H45	Germany
	VB 940345	Cattle	unknown	2011	Ont:H45	Germany
D	VB 992052	Cat	UTI	2009	O1:H6	Germany
	VB 970661.1	Dog	UTI	2010	O1:H6	Germany
	VB 970661.2	Dog	UTI	2010	O1:H6	Germany
	VB 985854	Dog	RTI	2011	O1:H6	Germany
	IMT13211	Horse	Metritis	2007	O1:H6	Germany
	VB 950030	Cat	UTI	2008	O1:H6	Germany
	VB 954112	Dog	UTI	2008	O1:H6	Germany
	VB 961231	Dog	STI	2008	O1:H6	Germany
	VB 990378.1	Dog	UTI	2008	O1:H6	Germany
	VB 988841	Dog	UTI	2010	Orough:H6	Italy
	VB 977451	Dog	UTI	2010	O1:NM	Germany
E	IMT16316	Black Bird	none	2008	Ont:H45	Germany
	IMT16343	Rock Pigeon	None	2008	Ont:H45	Germany
	IMT16352	Goose	None	2008	Ont:H45	Germany
	VB 933328	Cattle	Enteritis	2010	O45:H45	Germany
	IMT16357	Black Bird	None	2008	Ont:H45	Germany
	VB 964041.2	Horse	STI	2008	Ont:H45	Germany
	IMT21183	Human	UTI	2010	Ont:Hnt	Germany
	VB 962894.2	Dog	STI	2010	O1:NM	Italy
	WCE227	Human	UTI	2010	O25:H6	China
	IMT12560	Horse	STI	2007	O1:H6	Germany
	VB 967602.1	Dog	STI	2008	O1:H6	Germany
F	VB 992564	Cat	UTI	2010	Orough:H6	Germany
	VB 976972	Dog	UTI	2010	Orough:H6	Germany
G	YD28	Human	UTI	2006	O1:H6	USA
	EC7	Human	UTI	2006	O1:H6	USA
H	VB 998118.1	Cat	UTI	2010	O1:H6	Germany
	VB 998118.2	Cat	UTI	2010	O1:H6	Germany
I	VB 989726.1	Dog	UTI	2010	O1:NM	Italy
	VB 973559	Dog	STI	2009	O45:H45	Germany
	VB 958154	Horse	unknown	2009	Ont:H45	Germany
	VB 989188.3	Horse	STI	2010	nt	Germany
J	VB 976552	Dog	RTI	2009	O2:H42	Germany
	VB 962116	Horse	STI	2010	O2:H42	Germany
	VB 962089	Cat	UTI	2009	O2:H42	Germany
	VB 991720	Cat	RTI	2010	O2:H42	Germany
	IMT23463	Black Vulture	none	2010	O74:H42	Mongolia
	IMT23464	Black Kite	none	2010	O74:H42	Mongolia
S	VB 956098	Horse	STI	2010	Ont:H45	Germany

Fig. 18: Dendrogram showing the relationship of 47 ESBL-producing D-ST648 *E. coli* isolates based on XbaI-generated PFGE profiles. According to a similarity index of >85% *E. coli* strains are separated into ten PFGE clusters (A-J) and one singleton (S) isolate. Abbreviations: UTI = urinary tract infection; RTI = respiratory tract infection; STI = soft tissue infection; NM = non motile, nt = not typable; Color code: green = domestic animals; blue = human; red = wild bird.

Table 2: Summary of wildlife species tested, number of isolates and clones and rates of multi-resistant and/or ESBL-producing *E. coli* of this thesis.

Study	Host species	Rate of multi-resistant (MDR) and/or ESBL-producing isolates
(I) Antimicrobial resistance profiles of *E. coli* from common European wild bird species.	Barn Owl (*Tyto alba*), Blackcap (*Sylvia atricapilla*), Black-headed Gull (*Chroicocephalus ridibundus*), Blue Tit (*Cyanistes caeruleus*), Brambling (*Fringilla montifringilla*), Chaffinch (*Fringilla coelebs*), Common Buzzard (*Buteo buteo*), Chiffchaff (*Phylloscopus collybita*), Common Kestrel (*Falco tinnunculus*), Common Kingfisher (*Alcedo atthis*), Common Redstart (*Phoenicurus phoenicurus*), Common Swift (*Apus apus*), Common Treecreeper (*Certhia familiaris*), Dunnock (*Prunella modularis*), Egyptian Goose (*Alopochen aegyptiacus*), Eurasian Blackbird (*Turdus merula*), Eurasian Collared Dove (*Streptopelia decaocto*), Eurasian Coot (*Fulica atra*), Eurasian Jay (*Garrulus glandarius*), Eurasian Nuthatch (*Sitta europaea*), Eurasian Siskin (*Carduelis spinus*), Eurasian Sparrowhawk (*Accipiter nisus*), European Robin (*Erithacus rubecula*), European Starling (*Sturnus vulgaris*), Firecrest (*Regulus ignicapillus*), Rock Pigeon (*Columba livia*), Garden Warbler (*Sylvia borin*), Goshawk (*Accicepter gentilis)*, Grasshopper Warbler (*Locustella naevia*), Great Cormorant (*Phalacrocorax carbo*), Great Spotted Woodpecker (*Dendrocopos major*), Great Tit (*Parus major*), Greater White-fronted Goose (*Anser albifrons*), Green Woodpecker (*Picus viridis*), Greenfinch (*Carduelis chloris*), Grey Heron (*Ardea cinerea*), Grey Wagtail (*Motacilla cinerea*), Jackdaw (*Corvus monedula*), Long-eared Owl (*Asio otus*), Long-tailed Tit (*Aegithalos caudatus*), Mallard duck (*Anas platyrhynchos*), Marsh Warbler (*Acrocephalus palustris*), Mute Swan (*Cygnus olor*), Nightingale (*Luscinia megarhynchos*), Reed Bunting (*Emberiza schoeniclus*), Reed Warbler (*Acrocephalus scirpaceus*), Rook (*Corvus frugilegus frugilegus*), Serin (*Serinus serinus*), Song Thrush (*Turdus philomelos*), Tree Sparrow (*Passer montanus*), Whitethroat (*Sylvia communis*), White-throated Dipper (*Cinclus cinclus*), Willow Tit (*Poecile montanus*), Winter Wren (*Troglodytes troglodytes*), Yellowhammer (*Emberiza citrinella*)	8% MDR of 187 clones from 201 *E. coli* isolates (originating form 226 animals)
(II) Detection of pandemic B2-025-ST131 *Escherichia coli* harboring the CTX-M-9 extended spectrum ß-	Brown Rat (*Rattus norvegicus*)	1% ESBL of 211 clones from 238 isolates (originating form 87 animals)

lactamase type in a feral urban brown rat.		
Study	**Host species**	**Rate of multi-resistant (MDR) and/or ESBL isolates**
(IV) First insights into antimicrobial resistance among faecal *Escherichia coli* strains isolated from small wild mammals in rural areas.	Yellow-necked mouse (*Apodemus flavicollis)*, Wood mouse (*A. sylvaticus*), Striped field mouse (*A. agrarius),* Harvest mouse (*Micromys minutus*) and Norway rat (*Rattus norvegicus*)*,* family Cricetidae, i.e. Bank vole (*Myodes glareolus*, formerly *Clethrionomys glareolus*), European water vole (*Arvicola amphibius*), Common vole (*Microtus arvalis*), Field vole (*Microtus agrestis*), European pine vole (*Microtus subterraneus*), and different shrew species (order Soricomorpha or Eulipotyphla)	2.2% MDR of 188 clones from 188 isolates (originating from 1443 animals)
(V) CTX –M 15 type extended spectrum beta lactamases producing *E. coli* from wild birds in Germany.	Greater White-fronted Goose (*Anser albifrons*), Rock Pigeon (*Columba livia*), Eurasian Blackbird (*Turdus merula*)	2.3% ESBL from 172 isolates (originating form 172 animals)
(VI) Companion animals: a relevant source of extended-spectrum beta-lactamases producing, fluoroquinolone resistant *Citrobacter freundii*.	Tawny owl (*Strix aluco)*	1% ESBL from 172 isolates (originating form 172 animals)
(IX) Frequent Combination of Antimicrobial Multiresistance and Extraintestinal Pathogenicity in *Escherichia coli* Isolates from Urban Rats (*Rattus norvegicus*) in Berlin, Germany.	Brown Rat (*Rattus norvegicus*)	13.6% MDR from 211 clones from 238 isolates (originating form 87 animals)
(X) Comparable High Rates of Extended-Spectrum-Beta-Lactamase-Producing *Escherichia coli* in Birds of Prey from Germany and Mongolia.	Black Kite (*Milvus migrans*), Red Kite (*Milvus milvus*), Buzzard (*Buteo buteo*), Sea Eagle (*Haliaeetus albicilla*), Spotted Eagle (*Aquila pomarina*), Goshawk (*Accicepter gentilis*), Black Kite (*Milvus migrans*), Buzzard (*Buteo hemilasius*), Black Vulture (*Aegypius monachus*), Steppe Eagle (*Aquila nipalensis*), Golden Eagle (*Aquila chrysaetos*), Short-toed Eagle (*Circaetus gallicus*), Eurasian Hobby (*Falco subbuteo*), Kestrels (*Falco tinnunculus*), Saker Falcons (*Falco cherrug*), Lesser Kestrels (*Falco naumanni*), Demoiselle Cranes (*Anthropoides virgo*), Sandpipers (*Actitis hypoleucos*), Nightjar (*Caprimulgus europaeus)*, Hoepoe (*Upupa epops*)	**ESBL selective screening:** Germany: 13.8% ESBL of 65 isolates (originating from 171 animals, 5.2% of all animal carried ESBL-producing *E. coli*); Mongolia: 10.8% of 37 isolates (originating from 91 animals, 4.5% of all animals carried ESBL-producing *E. coli)*

(XI) Fecal carriage of Extended-spectrum beta-lactamase-producing *E. coli* in urban rats, a risk for public health?	Brown Rat (*Rattus norvegicus*)	**ESBL selective screening:** 19% ESBL of 42 clones from 120 isolates (originating from 56 animals, 16% of all animals carried ESBL-*E. coli*)

3. Discussion

ESBL-producing *E. coli* combine prototypic features of multi-resistant bacteria with a long tradition as an indicator bug for faecal pollution by Enterobacteriaceae. Therefore this "super-bug" presents an ideal paradigmatic organism for studying the spread of multi-resistant bacteria into the environment.

3.1. Avian and mammal host species of multi-resistant *Escherichia coli*

As summarized in Tab. 2 (p. 50-51), ESBL-producing *E. coli* carriage rates obtained in my studies were broadly diversified. In some of my early studies (**I, II, III, IV**) we did not use an initial selective screening for antimicrobial resistant *E. coli*. The reason for that was that we also wanted to isolate and characterize non-resistant but pathogenic *E. coli,* as there was and still is scarce knowledge on their occurrence in wildlife. The lower number or absence of ESBL-producing *E. coli* in the early "Rodent-study" (**IV**) might be due to a lack of those isolates by usage of non-selective media. This seems very possible, as some studies reported the occurrence of ESBL-producing *E. coli* in Central Europe in 2006 already [195]. The use of selective screening clearly enhances the sensitivity of detection of resistant isolates [196]. This fact clearly influenced my last studies (**X, XI**), in which we observed that the number of multi-resistant and ESBL-producing *E. coli* clearly rose. Certainly the usage of selective screening presents a closer approximation of the actual rates of multi-resistant *E. coli* in wildlife.

When looking at aspects of host species involved in the carriage of multi-resistant-*E. coli* in wildlife, general information on the microbiota of the host, especially on the carriage of Enterobacteriaceae, is crucial. Unfortunately data on the microbiota of wildlife is very limited and often restricted to single species as hosts of certain pathogens. Even the ubiquitous *E. coli* data on its occurrence in certain species is often limited to single studies. Where more studies

have been performed, as it is the case for birds, the degree of shedding varies a lot between different bird species [197].

The same has been shown for rodents and small mammals [137, 146]. The feeding behaviour of the host is also very important, as *E. coli* is most likely to be isolated from omnivore birds and mammals, as well as predators [197].

The host spectrum of *E. coli* itself therefore clearly influences the occurrence of multi-resistant *E. coli* in wildlife in my "host distribution study" **(I)**. Nevertheless this pilot study included the high number of fifty-five different species ranging from birds of prey to waterfowl to passerines, and we found that the first two avian groups were highly prevalent as carriers of multi-resistant *E. coli* isolates. Birds of prey in particular seem to constitute one of the major host groups of the resistant isolates. Parallel to our study, it was reported that avian predators can acquire resistant microorganisms via their prey [198].

This tertiary trophic level in the food chain may lead to accumulation events of multi-resistant bacteria and might be a possible explanation for their common detection. In this context it has been recently shown that resistant bacteria can spread from livestock to small wild animals in close proximity to farms [172], depicting a possible transmission route towards birds of prey. Another group associated with the carriage of multi-resistant *E. coli* was waterfowl, an involvement which is not surprising and most likely due to the uptake of such bacteria via contaminated water [140, 142, 199]. Although the current literature is heterogeneous in the classes of birds being detected as carriers of multi-resistant *E. coli,* birds of prey and waterfowl have often been found [147, 148, 150, 152-154, 157-162, 165-167, 200-202] (detailed species information is given in Table 1 of [**VIII**]). Interestingly, as not only waterfowl but also small passerines like Garden Warbler or White-throated Dipper from rural areas were present among the hosts of resistant bacteria, our study described the detection of multi-resistant *E. coli* in passerines for the first time. In the case of the Garden Warbler, an acquisition via the insectivorous lifestyle of the bird could be possible. Indeed, appearance of resistant bacteria on insects has already been described for cockroaches [203] and recently on flies in cattle farms [204].

The presence of pigeons among the hosts in our study supports the idea of a possible impact of synanthropism in the transmission of multi-resistant *E. coli*. The likelihood of bacterial

exchange between human sewage and birds has been found for gulls which shared strains of *E. coli* with isolates cultured from landfills and wastewater treatment plants [205].

Furthermore, it has been shown that the degree of synanthropic behaviour of the host animal species influences *E. coli* colonization. In other words, animals living in urbanized areas are more likely to carry *E. coli* than animals living in remote areas [96, 97, 206] thereby also influencing the carriage rates of multi-resistant *E. coli*.

In general, comparing my data to the current literature (refer to Tab. 1 of [**VIII**]), it clearly points out wild birds and rodents as one main group of wildlife carriers for multi-resistant *E. coli.* This might be explained by the high diversity of avian ecological niches and their ease in picking up human bacteria through waste. The importance might be influenced by the fact that research focused on birds and rodents because of the high carriage rates of multi-resistant *E. coli* in samples of early studies. However, these early influential studies also reported an absence or low numbers of multi-resistant *E. coli* in other groups of animals like deer, small ruminants, small and large predators, lagomorphae, reptiles,molluscs and amphibians [147, 156, 207].

The same seems to apply to the different ecological niches where these diverse avian species originated and water and human made habitats seem to be important. nevertheless due to complex scenarios like accumulation effects, also less human influenced areas seem to be involved. This might be partly due to the migratory behaviour of birds and their high mobility and living range, a fact that surely influenced another result of this study: we did not find any difference in the spatial distribution of multi-resistant bacteria from birds from different geographic backgrounds in Germany, as we detected approximately 5% of all birds as carriers of multi-resistant bacteria independent of their rural or urban origin. Comparable data on antimicrobial susceptibility of isolates from wild birds from Central Europe was at this time limited to Black-headed Gulls from the Czech Republic [142] and the resistance patterns of the isolates found in this study were comparable to those analysed by us.

The typing of the phylogenetic background and screening for virulence associated genes (VAGs) of the avian *E. coli* performed in a study of our group [208] revealed multi-resistant isolates belonging to phylogenetic groups linked with extra-intestinal pathogenicity like B2 or

D, which also harboured high numbers of VAGs. It was assumed previously that the phylogenetic lineage B2 consists of highly virulent strains [209].

Additionally, B2 and D strains have been described as "low resistance strains" in contrast to group A, and B1, which are more often found to bear resistance [210]. Our results from wildlife are in contrast to that common belief, and this observation of a combination of multi-resistance and virulence in *E. coli* isolates from wildlife was later underlined by the results of further studies both from our group and from others **(IX, X)**.

Unexpectedly for us, in the "Rodent study" **(IV)** which was set up to elucidate a possible link between avian predators and their prey, the main findings were low numbers of multi-resistant *E. coli* compared to the numbers obtained for avian hosts (2-3% vs. 5%). Although an influence of the sampling cannot be excluded, our own ongoing studies in the same population and the results of parallel studies by others in Canada and the Czech Republic support these findings [156, 172]. Despite the low numbers of antimicrobial resistant *E. coli*, an influence of synanthropic behaviour of rodents and/or the proximity to human infrastructures was found. All multi-resistant *E. coli* isolates of this study originated from rodents captured in close proximity to intensively used farmland.

Additionally, most of the rodent host species positive for carriage of multi-resistant bacteria are known to be synanthropic and agricultural pests (Wood mouse, Bank vole) [211], thereby having potential contact with antimicrobial resistant bacteria shed by livestock. This underlines data by others like Kozak *et al.* who detected resistant strains predominantly in rodents from swine farms [172], while Literak *et al*. [212] found that close contact of rodents with swine in farms is associated with transfer of resistance genes. Farms might therefore act as a source of rodents acting as vectors for antimicrobial resistance.

Additionally, similar to the results of the avian study (**I**) the resistance patterns of the rodent borne *E. coli* was comparable to the antimicrobial resistances found among *E. coli* from swine, poultry and cattle [213]. Also, the second interesting finding of this study supports this hypothesis, as we found a correlation between the carriage of multi-resistant *E. coli* isolates in rodents and livestock density indices for Germany ($p<0.05$).

This supports the idea of some kind of environmental pollution with multi-resistant *E. coli* by human farming practises and high livestock densities resulting in higher rates of multi-

resistant bacteria in rodents. This is in contrast to the findings of the avian study (**I**), where we did not find any differences in spatial distribution of multi-resistant *E. coli*.

Due to the limitation of the sampling scheme in terms of epidemiologic design and the overall low number of resistant *E. coli* isolates identified, this correlation has to be considered a preliminary finding. In conclusion, despite the low rates of antimicrobial resistance among wild rodents living in rural areas compared with other wildlife species in Central Europe [156, 165, 214], their role as an initial vector from farms to the environment should not be underestimated.

Clearly, rodents are not permanent reservoirs of resistant isolates but, via their role in the food chain, substantially contribute to an accumulation of multi-resistant bacteria in other species like birds of prey [156, 172].

Besides rural rodents, we also sampled an urban species: *Rattus norvegicus* (**IX**). The rates of multi-resistant *E. coli* determined in rats support the inevitable influence of anthropophily [143, 215] on the carriage rate of resistant bacteria in wildlife. They are in clear contrast to the rates of multi-resistant *E. coli* obtained from rural rodents (13.6% vs. approx. 2%), which is very likely due to urban rats´ association with human-created habitats like the sewage system, houses, gardens, farms and garbage dumps. In general, comparable data on antimicrobial resistance in *E. coli* from wild rats is rather limited.

Parallel to my study, Literak et al. (2009) identified 2.5% of African Black rat isolates (*R. rattus*) to be ESBL-producers [155]. An additional study reported high rates of multi-resistant *E. coli* in rats *(R. norvegicus)* from a port in Greece [216]. A comparison with other synanthropic wildlife species revealed that the rates of antimicrobial resistant *E. coli* detected in rats (26%) were higher than what has been found in raccoons (16% from urban environments) [86] or small mammals (15% in residential areas) [217]. As rats are the only mammal species that populates the sewage system directly, they have direct contact with human faeces, whether from private households or clinics and might frequently take up multi-resistant isolates via this route. The recent finding of comparable antimicrobial resistance patterns in *E. coli* isolates from rats and humans agrees with this [195, 218]. Up until now there is no data available concerning colonization of the rat´s gut with multi-resistant *E. coli* or the longitude of the shedding of these organisms. As this data is important for the risk assessment because it greatly influences the likelihood of re-infection of humans via rats, this remains an important research topic for the future.

3.2. Extended-Spectrum beta-lactamases producing *E. coli* in wildlife

Besides multi-resistant *E. coli,* we also detected ESBL-producing *E. coli* isolates in the studies discussed above which were characterized in more detail as ESBL-producing bacteria present one of the major challenges in infection medicine. This restriction due to practical and cost effectiveness reasons gave paradigmatic perceptions to their phylogenetic background, zoonotic nature and the thereof resulting overlaps to ESBL-producing *E. coli* isolates from human and veterinary medicine.

During the "Avian host study" (**I**) we detected a small but noticeable proportion of *E. coli* isolates, which were ESBL-producers (2.3%) (**V**). This study provided interesting insights as the four CTX-M-15-producing *E. coli* isolates showed identical genotypes (ST648) and macro-restriction patterns and therefore belonged to the same clone. This was of special interest, as they were recovered from four different bird species with different feeding characteristics, living in diverse habitats and sampled in a distance of up to 30 kilometres from each other. This means that certain ESBL-producing *E. coli* clones are either present in high numbers in certain areas and/or do not lack the fitness to survive in the environment to be picked up by different animals. The clone was therefore included in the last study (**XII**) of this habilitation thesis and will be discussed there.

The first description of an ESBL-producing *Citrobacter* in a wild animal (**VI**) underlines that, besides *E. coli,* other ESBL-producing enteric bacteria have found their way into the environment as well.

Besides wild avian hosts, mammals can also carry ESBL-producing *E. coli,* and the characterization of the rat-borne ESBL isolates led to the first description of the pandemic clonal group B2-O25b-ST131 of ESBL-producing *E. coli* in a wild mammal. At the same point in time, this clonal group had been observed for the first time in a wild bird by Hernandez [157]. As the strain was clonally related to human reference strains as well as to a number of ST131 companion animal isolates (**III**), it is obvious that urban rats carry zoonotic ESBL-producing *E. coli* strains implicating new urban transmission pathways [219]. As meanwhile several other studies reported the detection of ST131 in different wildlife species all over the world **(VIII)**, it has become obvious that ST131 is also successful outside clinical settings, which is an important finding and will be discussed later.

Putting my data into a global context, the occurrence of ESBL-producing *E. coli* in wildlife was recognized two decades after the first outbreaks in human clinical settings [51, 54, 147]. Interestingly, the spread of ESBL-producing *E. coli* into the environment was accompanied by the community onset of ESBL, leading to speculations whether environmental ESBL-producing *E. coli* are a spill-over form of environmental pollution from highly human influenced settings [29, 30, 95].

This theory is backed up by the fact that the first reports of ESBL-producing *E. coli* in wildlife date back to shortly after their appearance in livestock farming. This could also hint towards a manure-driven spread of ESBL-producing *E. coli* into the environment [111].

ESBL-producing *E. coli* from wildlife may thus express a multi-resistant phenotype, not due to the nearby use of antimicrobials or the presence of antimicrobials in sub-therapeutic concentrations in natural environments, but because distant use had caused a multi-resistant organism to evolve in the first place, which subsequently spread to different ecological niches [220]. Faecal contaminations, with their inevitable presence of commensal and pathogenic bacteria, can be assumed to be the most important link between settings with regular antimicrobial pressure (livestock farming, human and veterinary clinical settings), the environment and subsequently wildlife.

This would mean a constant release of antibiotic-resistant bacteria originating from human and animals into the environment through wastewater or manure [4]. The detection of antimicrobial resistant bacteria in aquatic environments affected by human and animal wastewater and soil provides evidence for this hypothesis [110].

From a geographical point of view, ESBL-producing *E. coli* isolates of wildlife origin have been reported worldwide including Europe [148-154, 156, 158-163, 165-167], Africa [155], Asia [157, 164] and North-America [221, 222] but so far not in Antarctica and Australia. The differences in the detection rates might reflect the different number of studies performed on these continents. Nevertheless, multi-resistant *E. coli* of wildlife origin have already been reported from continents with rare detection of ESBL-producers as well [131, 132, 135, 138, 140, 141, 172, 223]. Besides simple geographical attributes like the country or continent of origin, it seems more appropriate to reconsider the type of region where the isolates originates.

In this respect, important criteria for the classification of areas might include the natural preservation state, livestock density, type of livestock, animal management, as well as human density or the remoteness of an area in terms of intensity of human migration or travel in this area [96]. Furthermore, the detailed ecology of the host, including specificities like migration routes, breeding behaviour and social patterns have to be considered as well.

3.3. Carriage rates, pathogenicity and zoonotic potential of ESBL-producing *E. coli* from wildlife

To estimate the risk potential of ESBL-producing *E. coli* in wildlife, one of the first parameters that needs to be measured is the carriage rate in different wildlife species. As mentioned above, detection rates of resistant bacteria are greatly influenced by different factors like differences in the study design, use of selective media or the overall number of animals tested, altogether leading to a certain bias. Observed rates of ESBL-producing *E. coli* in different geographical areas ranged from 0.5% in birds of the remote Azores islands in the Atlantic Ocean [162] to 32% for birds of the Iberean peninsula [160]. The limitations that arise from this bias when interpreting these data certainly have to be kept in mind. Nevertheless, for Europe the number of studies performed on ESBL-producing *E. coli* in wildlife is high, and there does not seem to be a difference in the detection rates between agriculturally used lands or urban environments compared with natural preserve areas, since in both types of areas detection rates higher than 20 % have been observed (as reviewed in **VIII**). In Central Europe, even non-synantropic species like the Iberan wolf have been found as carriers of ESBL-producing *E. coli* [224]. For remote areas like the Azores or the Kamchatka peninsula the rates seem to be lower, with approximately 1% ESBL-producing *E. coli* [157, 162], suggesting dilution effects. In this context, unexpectedly for us, and in contrast to this data, in one of our studies we obtained equal numbers of ESBL-producing *E. coli* independent of the origin of the samples (from Germany or Mongolia).

The overall carriage rate of approximately 5% ESBL-producing *E. coli* furthermore indicates that ESBL-producing *E. coli* in wild birds are no occasional finding, even in remote areas in Mongolia.

This has been supported meanwhile by other studies (reviewed in **VIII**) [195]. Thus high carriage rates of ESBL-producing *E. coli* in some raptor seem to be commonly present, independent of the origin of the birds from natural preserved areas or farmland [195]. As we obtained high numbers of ESBL-producing *E. coli* for the Mongolian birds of prey, our data substantiate the theory of a contribution of avian migration to the transmission of multi-resistant *E. coli* into different environments [97, 195].

Keeping in mind the very remote conditions in the Mongolian Gobi desert, with no considerable human and livestock populations and the absence of agriculture or manure spread on fields, it is very unlikely that the raptors have taken up the ESBL-producing *E. coli* in Mongolia at the time of sampling. However, all Mongolian avian hosts sampled undergo southward winter migration, namely on the Korean Peninsula, to China and to India, connecting the remote Gobi desert to the globalized world with high carriage rates of ESBL-producing *E. coli* in human and livestock [32, 225-227]. This is exemplified by the occurrence of highly clonally related clinical relevant isolates in Mongolian birds and Belgian patient samples. The data presented in this study proved that the equation "no man, no resistance" is too simple, because non-human vectors of multi-resistant bacteria like birds need to be looked at as well [215]. The strong contribution of human international travel to the spread of antimicrobial resistance has only recently come into the spotlight [228]. Although its importance is often neglected, avian migration basically follows the same principles, and the number of migrating birds worldwide has been estimated to be five billion animals a year [229]. It is unlikely that geographically independent micro-evolution leads to the occurrence of identical clones in different areas of the world, and a transmission from one area to another is more probable. Of course human vectors have surely been involved in the transmission of these strains on their way to Mongolia, but, as these birds migrate the simplest explanation of the last step of transmission to this remote area is air borne spread. Compared to birds of prey we found alarmingly high rates of 16% of urban rats carrying an ESBL-producing *E. coli*. These rates exceeded those that have been recently reported for healthy individuals from comparable urban settings (5% - 8%) [230-233], but were similar to the ESBL-producing *E. coli* rates in hospitalized patients or their household contacts (12%- 16%) [234]. Rats from the sewer tunnels carried ESBL-producing *E. coli* twice as often (33%).

The high number of ESBL-producing *E. coli* determined among sewage tunnel rats indicates high ESBL-producing *E. coli* levels in the outflow of hospitals as the rats were trapped nearby the wastewater discharge of a large hospital. This points towards a permanent transmission of these bacteria from clinical environments to the rat population. Furthermore, we have first evidence for a vector function of urban rats in the transmission of ESBL-producing *E. coli* in urban environments.

We were able to trace one single clone in three different animals over a period of two months, originally appearing in the sewage system and finally recovered from a third animal in a nearby flat. This finding supports the idea of a spread of ESBL-producing *E. coli* from the sewage system to human infrastructures like flats by rats. As these rodents are assumed to populate urban areas by hundreds and thousands [235, 236], they might be a substantial permanent environmental source of zoonotic and multi-resistant bacteria.

Summing up the main finding concerning the carriage rates for avian and mammal ESBL hosts, it becomes obvious that clinically relevant ESBL-producing *E. coli* are present in high rates in urban rats and in lower degrees in birds of prey. Although there is a clear influence of the synanthropic behaviour of the animals as exemplified by the rats, even in remote areas the numbers are still inevitable. This finding underlines the utmost importance of the "One Health" concept and corroborates the urgent need for holistic approaches, comprising humans, animals and the environment, to explore putative transmission cycles of multi-resistant ESBL-producing *E. coli*. As a first step in this direction we performed a comparative analysis on ESBL-producing *E. coli* isolates of ST648 that we found twice in wild bird populations in Germany and Mongolia [166, 237](**V, X)**. First of all, the study underlined the importance of ST648 in terms of ESBL-carriage as we observed a rate for ST648 isolates among our companion collection that was comparable to the proportion of the major pandemic ESBL-clone ST131. Furthermore, by surveying the current literature it becomes clear that ST648 represents the second most often identified and published clonal group after ST131 among clinical ESBL-producing *E. coli.* Its importance can therefore hardly be denied [47]. The dissemination of distinct *E. coli* phylogenetic lineages, as exemplified by B2-ST131-CTX-M-15, supports the idea of an essential role of clonal spread of bacteria in the global expansion of ESBL-producing *E. coli* [63, 64, 68, 78, 79].

In this study (**XII**) we were able to show a similar situation for ST648 strains of human, domestic animals and wild avian origin, suggesting a successful spread of clinical ST648 ESBL-producing isolates into very different habitats and the existence of a second pandemic lineage of ESBL-producing *E. coli* besides B2-ST131-CTX-M-15. Our analysis of the clonal relatedness revealed that lineages that were present on different continents are clear evidence for a pandemic spread for ST648.

The screening for several virulence associated genes in our ST648 isolate collection found that, in comparison with the average number of VAGs observed for the B2-ST131 clone, the numbers of VAGs in ST648 were lower. This could be partly expected, as it is known that phylogenetic group D isolates commonly harbour fewer virulence genes than B2 isolates [182]. Nevertheless, as several typical ExPEC-VAGs were detected, the ST648 clonal group is likely to be virulent in human and animal infections too. ST648-*E. coli* might therefore have established what is already known for ESBL producing ST131 strains, namely a worrisome combination of multi-resistance and extra-intestinal virulence. Interestingly, this combination seems to apply for large proportions of the multi-resistant and/or ESBL-producing *E. coli* from all of my wildlife studies, as their characterisation showed phylogenetic backgrounds which are believed to be linked with high extra-intestinal virulence (ExPEC), like ancestral group B2 and D strains. Additionally, most of the isolates carried high numbers of genes associated with extra-intestinal virulence.

As mentioned above, exactly this combination of multi-resistance and virulence has been named as one of the reasons for the intercontinental spread of O25b:H4-ST131-CTX-15, which represents an ESBL–ExPEC [63]. There have been several attempts to explain the success of ST131 in clinical and non-clinical settings by its possession of virulence associated genes. This intrinsic virulence potential of ESBL-producing *E. coli* has been examined in a study by Branger et al. (2006) to clarify whether ESBL-producing *E. coli* represent traditional virulence clones of ExPEC or low-virulence opportunists whose ability to cause disease is largely limited to compromised hosts. Their basic outcome was that the emergence of ESBL-producing *E. coli* seems to be the result of complex interactions between the type of ESBLs, the genetic background of the strain, virulence associated genes and selective pressures in ecologic niches [28, 238, 239].

We tested one of our ESBL-producing *E. coli* from wildlife in an infection model as a paradigm for the *in vivo* pathogenicity of wildlife ESBL-producing *E. coli* and the combination of resistance and virulence. The urban rat-derived B2-ST95-O18:NM:K1-CTX-M-9 strain possessed a number of virulence genes, which confer adhesive, toxic and invasive properties and thus met all requirements for a successful commensal and extra-intestinal pathogenic life style, like ST131. And indeed, the chicken infection model proved that this hybrid strain not only had pathogenic potential but showed a high *in vivo* pathogenicity, even crossing the blood-brain barrier.

This finding contradicts previous assumptions of a burden of resistance [240-242] due to the carriage of ESBL -plasmids, as the carriage of an ESBL-plasmid did not interfere with the pathogenicity of the strain. It´s worth mentioning that such a combination, namely ESBL-producing ST95-*E. coli*, has only rarely been observed in human clinical samples so far, and its detection in wild rats, to the best of my knowledge is the first description of such a superbug in an animal. Although we showed it exemplary for one strain only, we can assume that the other ESBL-producing *E. coli* from wildlife that also had typical ExPEC-associated backgrounds and high numbers of virulence associated genes are very likely ExPEC strains with clinical relevance for animals and humans.

Furthermore, as we often encountered this combination of virulence with multi-resistance in wildlife isolates, this could be an explanation for their success in extra-clinical settings. As the possession of ExPEC typical genes has been associated not only with pathogenicity but also with the successful colonization of the mammalian gut [243], the combination with ExPEC typical genes might be one reason why multi-resistant strains are common in wildlife even in the absence of antimicrobial pressure.

Besides questions concerning carriage rates, another prerequisite for public health implication is a certain zoonotic character of ESBL-producing *E. coli* from wildlife. To help answer this basic question it is useful to address the population genetics of *E. coli*. One of the established ways to do so is to analyse data generated by MLST (www.mlst.net), which is believed to reflect the microevolution of the *E. coli* core genome.

Summing up data on STs of ESBL-producing *E. coli* from my review article (**VIII**), the MLST database (http://mlst.warwick.ac.uk/mlst/) and my own data on ESBL-producing *E. coli* and/or multi-resistant *E. coli,* we created a Minimum spanning tree (MSTree) based on MLST analysis, displaying the population structure of ESBL-producing *E. coli* (Fig. 19, p. 64). In general, this MLST analysis revealed that it is a rule rather than the exception that ESBL-producing *E. coli* belonging to identical sequence types are being isolated from different hosts, including human, domestic animals or wildlife. This indicates a common phylogeny and a clear zoonotic potential for most ESBL-producing *E. coli* so far [6]. At the moment, although the number of ESBL-producing *E. coli* from wildlife in global data sets is rather limited, the majority of the sequence types found in avian ESBL-producing *E. coli*, such as ST131, ST10, ST90, ST648 or ST167, are also present in human clinical isolates. If the same clusters of *E. coli* can cause disease in humans and domesticated birds, then transmission scenarios from and to wildlife are possible as well.

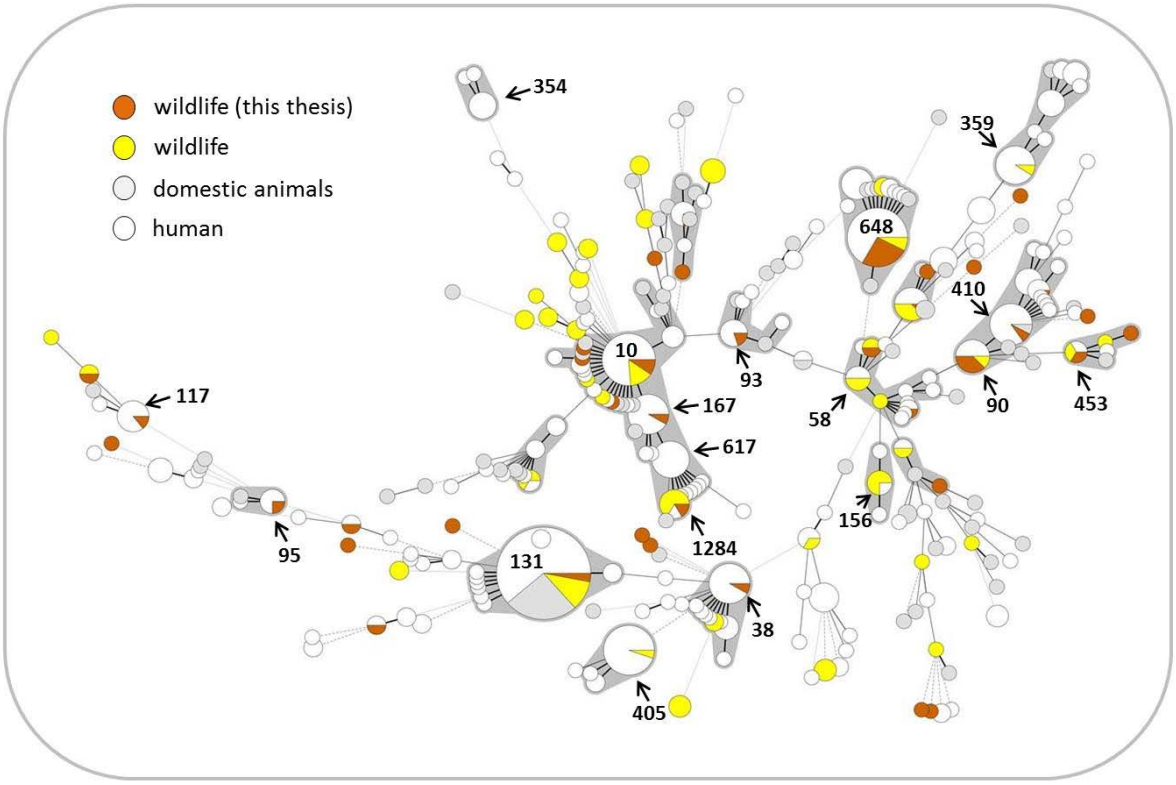


Fig. 19: Minimum spanning tree (MSTree) of wildlife, human, and domestic animal *E. coli* sequence types known for the production of ESBLs based on data used in my review (**IX**) and the MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli); orange: ESBL and multi-resistant *E. coli* STs identified in my wildlife studies, yellow: wildlife studies from others, white: human isolates, grey domestic animals; highlighted in grey: Sequence type complexes, calculated with Bionumerics 7.6 (Applied Maths, Belgium).

Besides the clonal spread of ESBL-producing bacteria, the plasmidal transmission is of equal importance.

The spectrum of the different enzyme types of ESBLs that I found in my wildlife studies and that has been reported by others is quite narrow compared to clinical isolates of human and veterinary origin [28, 69] (**VIII**). The reasons for this are unknown but several hypotheses seem possible. First, the narrow range of ESBL-genes found in wildlife might simply reflect the small number of studies performed on wildlife so far. Additionally another possible explanation is that the types of ESBLs found in wildlife are simply the most prevalent in human and veterinary clinics and in livestock farming, such as $bla_{\text{CTX-M-1}}$ or $bla_{\text{CTX-M-15}}$ [28, 69] and that the situation we observe in wildlife just presents spill-over effects from clinics and livestock farming. The high overall similarity of wild animal isolates with human or veterinarian clinical isolates supports this hypothesis [151, 156, 214]. However, as the dissemination of ESBL-genes is highly driven by horizontal gene transfer through plasmids, the occurrence of identical ESBL-genes could also be based on the spread of ESBL-plasmids which are randomly distributed in the environment.

Another explanation could be that certain types of plasmidal beta-lactamases are more successful in the environment due to co-selection events of other non-resistance genes accompanied by these beta-lactamases on the same plasmid. The selection advantage "resistance" might here be of minor importance and other up until now unknown effects might pose a survival advantage for *E. coli* carrying these plasmids.

Conclusions and Perspective

The basic outcomes of my work on ESBL-producing *E. coli* in wildlife can be summarized into five main aspects that should be implemented in future research:

(i) the data perfectly underline the utmost importance of the "One Health" aspect

(ii) my results raise the question of whether spill over of resistant bacteria from areas of high antibiotic usage or ubiquitous sub-therapeutic concentrations of antimicrobials in the environment are responsible for the common occurrence of ESBL-producing *E. coli* in wildlife

(iii) the data indicate the suitability of wild birds as sentinels for the environmental pollution by multi-resistant faecal bacteria

(iv) my results underline the necessity of combining two classical fields of natural-science, ornithology and medical microbiology, to reveal the impact of bird migration on the spread of multi-resistant bacteria into the environment

(v) in the absence of antimicrobial selective pressure, mechanisms other than resistance seem to contribute to the success of multi-resistant bacteria

My past research on ESBL-producing *E. coli* in wildlife revealed a widespread and substantial carriage of these multi-resistant strains in certain wild animal populations like waterfowl, birds of prey and rodents, even though these have never been exposed continuously to antibiotics. Furthermore, my data clearly show that the strains which can be found in wildlife are basically the same that have been found in the clinical field, be it in human or veterinary medicine, underlining the zoonotic character of ESBL-producing *E. coli.* My work and that of others helped therefore to put the environment and especially wildlife on the list for holistic approaches in the epidemiological research on antimicrobial resistance. This work is in line with major statements of the "One Health" initiative, which aims at expanding interdisciplinary collaborations and communications in all aspects of health care for humans, animals and the environment (http://www.onehealthinitiative.com/about.php).

Multi-resistant bacteria from wildlife are a paradigm for the “One Health” approach, as they bridge the gap from microbial ecology over environmental health and veterinary medicine to molecular microbiology, human medicine and health economics.

The implications for public health and the ecological consequences are unforeseeable at the moment. Wildlife as a source of multi-resistant bacteria has the potential to serve as an environmental reservoir and melting pot of bacterial resistance. Future studies should pursue this work to further unravel the role of wildlife in the cycle of transmission and spread of antimicrobial resistant *E. coli* between domestic animals like livestock and companion animals, humans and the environment. This requires interdisciplinary work and the collaboration of wildlife ecologists or ornithologists as well as human and veterinary clinicians. Additionally, experts from the field of waste management and water resources are required for the set up of studies alongside the possible routes of transmission of multi-resistant bacteria into the environment and into wildlife and possibly back to humans or domestic animals.

Another basic outcome of my work and the research of other groups is the need for epidemiologically well-founded studies on the spread of antimicrobial resistance in wildlife. The work that has been done up until now reveals that ESBL-producing *E. coli* are clearly a problem in wildlife, but presently only small scale studies with limited numbers of animals have been performed. Based on my results, the question arises whether spill over of resistance from areas of high antibiotic usage or ubiquitous sub-therapeutic concentrations of antimicrobials in the environment are responsible for the common occurrence of ESBL-producing *E. coli* in wildlife. Overall, the common occurrence of ESBL-producing *E. coli* in wildlife weakens the presumption that resistance will decline with the absence of antibiotic treatment alone, but underlines the very complex nature of its spread. Although some of the species tested during my previous work (like the urban rats) might be exposed in intervals to sub-inhibitory concentrations of antimicrobials, for wild birds in remote areas this is certainly rather an exception. The selection mechanisms responsible for maintaining high prevalence of resistance even in the absence of selective antimicrobial pressure are largely unknown and therefore need to be addressed more soundly [134].

Interestingly, most of my wildlife ESBL-producing *E. coli* combined multi-resistance with extraintestinal virulence properties, which could be one explanation for their presence in wildlife, as virulence features might present selection advantages.

Future epidemiological studies should aim at revealing the source and the reason of multi-resistant bacteria in wildlife to verify the impact of spill over from areas of high usage of antimicrobial substances compared to effects of sub-therapeutic concentrations in the environment. Studies alongside gradients of antibiotic usage would be helpful to investigate the effects of spill-over. In addition, they should combine the characterization of resistant bacteria in terms of the importance of clonal spread of multi-resistant bacteria vs. plasmidal spread of resistance genes with a sound determination (e.g. via Liquid chromatography–mass spectrometry [LC-MS]) of the concentration and range of antimicrobials and their metabolites in the environment where the resistant bacteria were isolated. This can be done on different animated/in-animated systems like (I) the explicit synanthropic combination health care facilities/urban waste water/rats to reveal possible urban transmission pathways, as the chances of transmission are higher due to closeness of wildlife and humans in cities, or (II) on farms and their proximate environment including livestock and species often related to pens like mice, flies, swallows and owls and environmental samples including feed, soil, dust, or manure and (III) on a global scale on intercontinental migratory birds and environmental samples including water, soil and their feed in their hibernation places and transit stops between areas of high usage to remote areas with low human influence.

Furthermore, certain wildlife species investigated during my studies have the potential to be used as sentinels to monitor the spread of antimicrobial resistant bacteria into the environment. Birds of prey (*Buteo* sp. *Milvus* sp.) and waterfowl species (*Anas* sp. *Anser* sp.) are well suited for different sentinel purposes. For both groups of birds long range migration is well known and has been studied for decades. Thus, intercontinental spread of resistant bacteria would be detectable. Furthermore, certain species of these groups occur worldwide and both groups of host species carry *E. coli*, which has been used as indicator for faecal pollution for decades as well.

Due to their place on top of the food chain, birds of prey might reflect the overall environmental resistance situation, whereas waterfowl seem to mirror the environmental pollution in rivers and lakes. Due to bird ringing, the migration behaviour of these avian groups is known in great detail. This allows comparisons of long range and short range migrating populations with locally hibernating birds and their different ecological niches.

Additionally, my work contributed to what has been called “microbial ornithology” as “two traditional disciplines came together to chart the movements and roles of wild birds in disseminating multi-resistant bacteria” (Bernard Dixon, Microbe 12, May, 2013) [244].

While international travel by humans has long been recognized as contributing to the dissemination of pathogens, interest in its role in spreading multi-resistant bacteria has attracted attention only recently [245]. Even more surprising, given that an estimated 5 billion birds migrate each year, is the current disregard of avian migration as a vehicle for the global dissemination of resistance.

Many different bird species, including those that were already identified as carriers of ESBL-producing *E. coli,* display considerable mobility, often involving the crossing of continents. In the same way, the phenomenon of bird migration creates the potential for the establishment of new endemic foci of disease along their routes. Similar to the spread of the West Nile Virus in the USA [245], antimicrobial resistant bacteria might also be carried over long distances by avian hosts. Bird migration could therefore contribute to the dissemination of resistance over the globe as has previously been observed for human travellers [246, 247].

To characterize the influence of bird migration, international and interdisciplinary collaborations alongside known migration routes of the above-mentioned sentinel species like *Milvus* sp. or *Anser* sp. are needed, involving ecologists, bird ringers and microbiologists.

Besides these grass root epidemiology approaches, two other future research topics can be developed from my data: First of all, we need to answer basic questions in terms of bacteria-host interactions. At this point we do not know if ESBL-producing *E. coli* colonize the gut of different animals more successfully than non-ESBL-producers, or whether they are just transient strains.

In the case of a stable colonization and subsequent shedding of ESBL-producing *E. coli,* wildlife could have the potential to re-infect human and domestic animal populations. Even for domestic animals, data on the shedding rates of multi-resistant bacteria and details of the interaction with the commensal gut microbiota are scarce. Currently, we do not know if certain ESBL-producing *E. coli* lineages might even have advantages compared to the non-resistant strains in the colonization of the gut, even in the absence of constant antimicrobial selections pressure or under sub-inhibitory concentrations. The theory of a burden of

resistance which has been put forward over the last decades must be re-evaluated, as some of my results and the results of others indicate that this burden might be of overestimated importance, at least for some certain multi-resistant *E. coli* lineages [3, 65, 248]. Therefore, we need fundamental molecular research on the question of why ESBL-producing *E. coli* do not perish in the environment and seem to be successful outside clinical settings. Summing up all available data, it becomes obvious that ESBL-producing *E. coli* are no longer solely a problem of hospitals, veterinary clinics or livestock breeding, where we can surely expect antimicrobial selection pressure helping resistant bacteria to survive. Their presence in wildlife and other environmental sources supports the hypothesis that non-resistance factors of certain ESBL-producing *E. coli* lineages contribute to their spread in different habitats, including clinical settings as well as the environment.

There is a bundle of possibilities of how non-resistance factors can influence the transmission of ESBL-producing *E. coli*, and we have to keep in mind the two general ways ESBL-producing *E. coli* could spread into the environment: (i) a plasmid driven spread or (ii) the dissemination of clonal lineages or, in other words, the whole bacterium. Most of the ESBL-genes like $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and bla_{OXA} are encoded on plasmids, which can be rapidly transmitted between different bacterial species, and a broad and fast dissemination in the environment seems likely. However, plasmid-driven spread cannot explain why certain phylogenetic lineages (sequence types, STs) like ST131, ST648, ST410, ST167 or ST405 are associated with ESBL production and dominate the literature on ESBL-producing *E. coli* [249]. These "ESBL associated sequence types (ST)" support the theory that the host of the plasmid--the bacterium itself--also plays an important role. As ESBL-plasmids are a part of the accessory genome, they should be equally distributed over the whole *E. coli* population and not predominantly in certain sequence types.

A high diversity of inc/rep types has been found in different ESBL-associated sequence types. Consequently, it is unlikely that the incompatibility group alone determines the combination of ESBL-plasmid with a certain ST. It therefore seems that besides plasmids the phylogenetic background is important for the dissemination of successful ESBL-producing *E. coli*. This is certainly true for B2-O25b:H4-ST131-CTX-M-15 and my data and that of others further points toward a similar situation for ESBL-producing *E. coli* of ST648.

Besides these mentioned data, the unpublished results of our group on MLST data of more than 1000 *E. coli* isolates including ESBL-producing strains furthermore substantiates this hypothesis, as the diversity index (Simpson´s Index of diversity [250]) of ESBL-producing isolates originating from phylogroups D und B2 was very low, pointing toward an accumulation of ESBL-producing *E. coli* in certain sequence types within these EcoR groups. One explanation for this observation is a specific interaction of ESBL-plasmids with the phylogenetic background, which has already been reported for the rare *E. coli*-phylotyp D2 [20] and has been discussed as a kind of convergent evolution [251].

The existence of "ESBL associated sequence types" raises the question of the reasons for their presence as, like mentioned above, plasmids as part of the accessory genome should be equally distributed over the whole phylogenetic background. Future research should therefore focus on the interaction between bacterial host and ESBL-plasmids. Such interaction could result in higher virulence or better adaptation to specific habitats and therefore explain the success of particular clonal lineages. In fact, for ESBL-producing *Klebsiella pneumoniae* strains, an influence of ESBL-plasmids on the gene expression of fimbrial adhesins leading to the complex changes in the invasion ability of *Klebsiella* strains into epithelial cells has already been shown [252]. In that light, several explanations for the success of ESBL-producing *E. coli* in different habitats are possible, and as ST131 or ST648 can be found both in clinical and extra-clinical settings like wildlife or the environment, they seem like good candidates for paradigmatic detailed characterization of their genetic backbone, as well as the plasmids they are carrying.

My future research will therefore focus on features like uptake rates of plasmids by certain phylogenetic lineages, interaction of the core genome with plasmids and the influence of low dose antimicrobial pressure on the persistence of plasmids.

Furthermore, the genetic makeup of the successful "ESBL associated sequence types" ST648 and ST131 will be investigated by whole genome sequencing and compared to other phylogenetic lineages to search for chromosomal genetic markers which might lead to advantages in general fitness, metabolism, adhesion or motility or compensation mechanisms for plasmid carriage. This could help to answer the question of why certain "ESBL-associated sequence types" are so successful in different habitats with or without constant antibiotic pressure. This data should also help to estimate if ESBL-plasmids bring along more features

than only resistance and if the theory of a burden of resistance can still hold for certain lineages of multi-resistant bacteria.

Abbreviations

CLSI	*Clinical and Laboratory Standards Institute*
CTX-M	*Cefotaximase-Munich beta-lactamase*
EcoR	*Escherichia coli Reference Collection*
EHEC	*enterohemorrhagic Escherichia coli*
EPEC	*enteropathogenic Escherichia coli*
ExPEC	*extra-intestinal pathogenic Escherichia coli*
IMT	*Institute of Microbiology and Epizootics*
inc/rep	*incompatibility/replicon type*
InPEC	*intestinal pathogenic Escherichia coli*
kb	*kilobases*
LU	*livestock unit*
MIC	*minimal inhibitory concentration*
µg	*microgramm*
MLST	*Multilocus sequence typing*
MLV	*multi locus variant*
MRSA	*Methicillin resistant Staphylococcus aureus*
NDM	*New Dehli beta-lactamase*
NM	*non motile*
nt	*non typeable*
OXA	*Oxacillinase beta-lactamase*
PCR	*Polymerase chain reaction*
PFGE	*Pulsed field gel electrophoresis*
RAPD	*Randomly amplified polymorphic desoxribunucleic acid*
RTI	*respiratory tract infections*
SHV	*Sulfhydryl variable beta-lactamase*
SLV	*single locus variant*
sp.	*species*
ST	*Sequence type*
STI	*soft tissue infections*
TEM	*Temoneira beta-lactamase*
UTI	*urinary tract infection*
VAGs	*virulence associated genes*
VRE	*Vancomycin resistant Enterococci*

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Curriculum Vitae

For reasons of data protection,

the curriculum vitae is not included in the online version

Publications

Description of the contributions to the publications used in this thesis

To keep this part focussed, only the main contributors for each part (idea and design, practical work, data analysis, writing) are mentioned.

(I) Guenther S, Grobbel M, Lübke-Becker A, Goedecke A, Friedrich ND, Wieler LH, Ewers C, **Veterinary Microbiology** 2009, 144(1-2): 219-25, Antimicrobial resistance profiles of *E. coli* from common European wild bird species. http://dx.doi.org/10.1016/j.vetmic.2009.12.016

Idea and Design:	**Guenther**	Data analysis:	Guenther, Ewers
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(II) Guenther S, Grobbel M, Beutlich J, Guerra B, Ulrich RG, Wieler LH, Ewers C, **Journal of Antimicrobial Chemotherapy** 2010, 65: 582-584, Detection of pandemic B2-025-ST131 *Escherichia coli* harboring the CTX-M-9 extended spectrum ß-lactamase type in a feral urban brown rat (*Rattus norvegicus*). http://dx.doi.org/10.1093/jac/dkp496

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(III) Ewers C, Grobbel M, Stamm I, Kopp P, Diehl I, Semmler T, Fruth A, Beutlich J, Guerra B, Wieler LH, Guenther S, **Journal of Antimicrobial Chemotherapy** 2010, 65: 651-660, Emergence of human pandemic O25:H4-ST131 CTX-M-15 ESBL producing *Escherichia coli* among companion animals. http://dx.doi.org/10.1093/jac/dkq004

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(IV) Guenther S, Grobbel M, Heidemanns K, Schlegel M, Ulrich RG, Ewers C, Wieler LH, **Science of the total Environment** 2010, 408(17): 3519-22, First insights into antimicrobial resistance among faecal *Escherichia coli* strains isolated from small wild mammals in rural areas.

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(V) Guenther S, Grobbel M, Beutlich J, Bethe A, Friedrich ND, Goedecke A, Lübke-Becker A, Guerra B, Wieler LH, Ewers C, **Environmental Microbiology Reports** 2010, 2(5): 628–705, CTX –M 15 type extended spectrum beta lactamases producing *E. coli* from wild birds in Germany.

http://dx.doi.org/10.1111/j.1758-2229.2010.00148.x

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(VI) Ewers C, Bethe A, Wieler LH, Guenther S, Stamm I, Kopp P, Grobbel M, **International Journal of Antimicrobial Agents** 2011, 37(1): 86-7, Companion animals: a relevant source of extended-spectrum beta-lactamases producing, fluoroquinolone resistant *Citrobacter freundii*. http://dx.doi.org/10.1016/j.ijantimicag.2010.09.007

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(VII) Ewers C, Grobbel M, Bethe A, Wieler LH, Guenther S, **Berliner Münchner Tierärztliche Wochenschrift** 2011, 124: 10–17, Extended-spectrum beta-lactamases-producing Gram-negative bacteria in companion animals: action is clearly warranted!

http://vetline.de/open-access-esbl-companion-animal-antibiotic-multi-resistance-surveillance/150/3130/69156

Idea and Design: Wieler, Guenther, Ewers Data analysis: Wieler, Guenther, Ewers

Writing: Wieler, Guenther, Ewers

(VIII) Guenther S, Ewers C, Wieler LH, **Frontiers in Antimicrobial Resistance and Chemotherapy** 2011, 2:246. Extended-spectrum beta-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution? http://dx.doi.org/10.3389/fmicb.2011.00246

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(IX) Guenther S, Bethe A, Fruth A, Semmler T, Ulrich RG, Wieler LH, Ewers C, **PLoS ONE** 2012, 7(11): e50331, Frequent Combination of Antimicrobial Multiresistance and Extraintestinal Pathogenicity in *Escherichia coli* Isolates from Urban Rats (*Rattus norvegicus*) in Berlin, Germany. http://dx.doi.org/10.1371/journal.pone.0050331

Idea and Design: **Guenther**, Ewers Data analysis: **Guenther**, Ewers

Practical Work: **Guenther**, **Ewers** Writing: **Guenther**, Ewers

(X) Guenther S, Aschenbrenner K, Stamm I, Bethe A, Semmler T, Stubbe A, Stubbe M, Batsajkhan N Doi Y, Wieler LH, Ewers C, **PLoS ONE** 2012, 7(12): e53039, Comparable High Rates of Extended-Spectrum-Beta-Lactamase-Producing *Escherichia coli* in Birds of Prey from Germany and Mongolia. http://dx.doi.org/10.1371/journal.pone.0053039

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(XI) Guenther S, Wuttke J, Bethe A, Vojtěch J, Schaufler K, Semmler T, Ulrich RG, Wieler LH, Ewers C, **Antimicrobial Agents and Chemotherapy** 2013, 57(5):2424-5, Fecal carriage of Extended-spectrum beta-lactamase-producing *E. coli* in urban rats, a risk for public health?

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http://dx.doi.org/10.1128/aac.02321-12

(XII) Ewers C, Bethe A, Grobbel M, Stamm I, Kopp PA, Guerra B, Stubbe M, Doi Y, Zong Z, Kola A, Semmler T, Schaufler K, Fruth A, Wieler LH, Guenther S **Journal of Antimicrobial Chemotherapy** 2014, 69(5):1224-30, CTX-M-15-D-ST648 *Escherichia coli* from companion animals and horses: another pandemic clone combining multiresistance and extraintestinal virulence? http://dx.doi.org/10.1093/jac/dkt516

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Practical Work:	Ewers, Bethe, Guenther	Writing:	Guenther, Ewers

Erklärung

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Dr. Sebastian Günther

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