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

Molecular analysis of ESBL-producing *Escherichia coli* from different habitats discloses insights into phylogeny, clonal relationships and transmission scenarios

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Katharina Anna Christina Schaufler
Tierärztin aus Nürtingen

Berlin 2016

Journal-Nr.: 3873

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Prof. Dr. Lothar H. Wieler
Zweiter Gutachter:	PD Dr. Sebastian Günther
Dritter Gutachter:	Univ.-Prof. Dr. Thomas Alter

Deskriptoren (nach CAB-Thesaurus):
dogs, birds, Escherichia coli, extended spectrum betalactamases, antibiotics, drug resistance, genome analysis, base sequence (MeSH), wildlife animals, public health, berlin

Tag der Promotion: 13.06.2016

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-732-3
Zugl.: Berlin, Freie Univ., Diss., 2016
Dissertation, Freie Universität Berlin
D 188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

FIGURES

TABLES

LIST OF ABBREVIATIONS

adk:	Gene for adenylate kinase
AmpC:	Ampicillinase C
AMR:	Antimicrobial resistance
APEC:	Avian pathogenic *E. coli*
bla:	Gene for beta-lactamase
CLSI:	Clinical and Laboratory Standards Institute
CRE:	Carbapenem-resistant Enterobacteriaceae
CTX:	Cefotaximase beta-lactamase
DNA:	Desoxyribonucleic acid
EAEC:	Enteroaggregative *E. coli*
EcoR:	*E. coli* reference collection
***E. coli*:**	*Escherichia coli*
EDTA:	Ethylenediaminetetraacetate
e.g.:	For example
EHEC:	Enterohemorrhagic *E. coli*
EIEC:	Enteroinvasive *E. coli*
EPEC:	Enteropathogenic *E. coli*
ESBL:	Extended-spectrum beta-lactamase
et al.:	And other people
ETEC:	Enterotoxigenic *E. coli*
ExPEC:	Extra-intestinal pathogenic *E. coli*
fumC:	Gene for fumarate hydratase C
gyrB:	Gene for DNA gyrase B
HTS:	High-throughput sequencing
icd:	Gene for isocitrate dehydrogenase
IMT:	Institute of Microbiology and Epizootics
InPEC:	Intestinal pathogenic *E. coli*
MALDI-TOF:	Matrix-assisted laser desorption/ionization-time of flight
mdh:	Gene for malate dehydrogenase
MLEE:	Multi-locus enzyme electrophoresis
MLST:	Multi-locus sequence typing

MRSA:	Methicillin-resistant *Staphylococcus aureus*
NGS:	Next-generation sequencing
NMEC:	Neonatal meningitis *E. coli*
OXA:	Oxacillinase beta-lactamase
PBP:	Penicillin binding protein
pCMB:	4-Chloromercuribenzoic acid
PCR:	Polymerase chain reaction
PFGE:	Pulsed-field gel electrophoresis
purA:	Gene for adenylosuccinate synthetase
RAPD:	Randomly amplified polymorphic DNA
recA:	Gene for recombinase A
RFLP:	Restriction fragment length polymorphism
rRNA:	Ribosomal ribonucleic acid
SePEC:	Septicemic *E. coli*
SHV:	Sulfhydryl variable beta-lactamase
SNP:	Single-nucleotide polymorphism
spp.:	Species plural
ST:	Sequence type
TEM:	Temoneira beta-lactamase
UPEC:	Uropathogenic *E. coli*
VRE:	Vancomycin-resistant *Enterococcus*
WGS:	Whole-genome sequencing

1. INTRODUCTION

One Health describes the worldwide collaborations, communications and actions in the human, animal and environmental sectors to address important public health challenges (Fig. 1) [1]. Antimicrobial resistant bacteria are among these major global health challenges affecting both human and veterinary medicine as well as environmental health [2]. Zoonotic extended-spectrum beta-lactamase (ESBL)-producing multi-resistant *E. coli* exemplify this threat. These versatile bacteria are not only commensals but the cause of severe intestinal and extra-intestinal diseases such as diarrhea [3], bloodstream [4] and urinary tract infections [5, 6] and due to the limitations in antimicrobial therapies increasingly difficult to treat [7]. They are not restricted to human and veterinary clinical contexts but emerge increasingly in extra-clinical settings such as the human community [8-10], wildlife [11-14] and the environment [15-17], presenting important opportunities to study phylogenetic and clonal relationships and transmission scenarios.

Figure 1: The One Health concept addresses the relationships of human, animal and environmental health to fight global health challenges such as antimicrobial resistance.

Numerous studies have dealt with the characterization of clinically relevant ESBL-producing *E. coli* from humans [8, 9, 18-25] and domesticated animals [26-34], congruently reporting rising detection rates. Less data from the environment and wildlife are available, with most studies having focused on aquatic ecosystems [35, 36], wild birds [12, 37-39] and rodents [13, 40]. The aim of this thesis has been to contribute to the characterization of ESBL-producing *E. coli* in rather unexplored environmental settings and to reveal phylogenetic and clonal relationships with a subsequent statement on transmission scenarios of isolates across different habitats and hosts.

The thesis addressed:

i) The detection rate and molecular characterization of ESBL-producing *E. coli* in dog feces collected from the extra-clinical environment

ii) The clonal and phylogenetic comparison of ESBL-producing *E. coli* isolates of dog feces from the environment (i) and canine clinical samples of a small animal clinic in the same adjacent area

iii) The detection rate and molecular characterization of ESBL-producing *E. coli* from wild birds

iv) The clonal and phylogenetic comparison of selected ESBL-producing *E. coli* isolates of sequence type (ST) 410 from dog feces (i), canine (ii) and human clinical samples and wild birds (iii) from the same geographic region

To achieve these objectives, different molecular characterization methods for *E. coli* ranging from clonal macrorestriction analysis and subsequent pulsed-field gel electrophoresis, through multi-locus sequence typing and bioinformatics tools based on bacterial whole-genome sequence data were applied. Issues **i** and **ii** were addressed in publication **I** ("Putative connection between zoonotic multiresistant extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in dog feces from a veterinary campus and clinical isolates from dogs" [41]) and those indicated **iii** and **iv** were dealt with in publication **II** ("Clonal spread and interspecies transmission of clinically relevant ESBL-producing *Escherichia coli* of ST410 – another successful pandemic clone?" [42]).

2. LITERATURE

To lead the reader to an overall better understanding of antimicrobial resistant ESBL-producing *E. coli* from different hosts and settings and their characterization possibilities, this literature survey aims to convey different topics. It is divided into five sections, starting with the first chapter on the worldwide importance of antimicrobial resistance, beta-lactam antimicrobials and ESBL-enzymes, followed by a general introduction to *E. coli*. The third and fourth chapters address the occurrence of ESBL-producing *E. coli* in different hosts and habitats, including humans and animals as well as environmental settings, highlighting their zoonotic potential and role in the context of One Health. The fifth chapter deals with aspects of *E. coli* characterization methods including multi-locus sequence typing (MLST), macrorestriction analysis and bioinformatics tools to elucidate phylogenetic and clonal relationships as well as the transmission potential of ESBL-producing *E. coli.*

Throughout this doctoral thesis, ESBL-producers are defined according to the CLSI guidelines M31-A3 [43], thus detected positive on a confirmatory test differing in the inhibition zones of a third-generation cephalosporin alone and in combination with a beta-lactamase inhibitor of over 5 millimeter, for a subsequent AmpC-producer exclusion.

2.1 Antimicrobial resistance (AMR) and beta-lactam antimicrobials

- AMR BACTERIA ARE AN INCREASING WORLDWIDE CHALLENGE
- RESISTANCE AGAINST BETA-LACTAMS PLAYS A MAJOR ROLE IN HUMAN AND VETERINARY MEDICINE

Antimicrobials are an essential keystone of modern medicine not only in terms of infection treatment but also for intensive care, oncology, neonatology and transplant medicine [44, 45]. The ongoing emergence of AMR bacteria, viruses, fungi and parasites causing ever-increasing infectious diseases attracts attention all over the world and is widely considered a severe medical and public health threat [46]. It limits the prevention and treatment possibilities of infectious diseases considerably [46]. AMR occurs if the minimal inhibitory concentration for a microorganism is so high that the approved standard and once effective dosage can no longer treat an infection caused by this agent.

AMR bacteria, in particular, play a key role in a new era in which infections and minor injuries might be no longer treated properly, subsequently leading to a negative effect on

patient outcomes and health expenditures [46]. Many bacteria are not only resistant to one but to at least three classes of antimicrobials, thus showing a multi-resistant phenotype [47].

While the widespread application of anti-infectives in human and veterinary medicine crucially influenced the selection of bacterial resistance [48], findings have demonstrated antimicrobial resistances even before the respective antimicrobial was introduced as a therapeutic: “antibiotic resistance is ancient” [49]. A bacterial penicillinase, for instance, was discovered several years before penicillin was first commercialized [50, 51]. Clinically relevant and environmental bacteria share a large proportion of their AMR determinants (resistome) [52, 53]. The acquisition of these determinants by clinical pathogens from extra-clinical bacteria is mainly driven by horizontal gene transfer and influenced by the broad application of antimicrobials in the clinics [50].

AMR in bacteria is classified in primary and secondary resistances. Primary, in general chromosomally encoded resistances are genus or species specific, whereas secondary resistances are strain specific and acquired, for instance through mutation and subsequent selection or via horizontal gene transfer [54]. Important resistance mechanisms are enzymatic destruction or modification of the antimicrobial substance, target structure changes and active removal of the component from the cell [54].

2.1.1 Beta-lactam antimicrobials

Anti-infectives changed medicine in the 20th century profoundly and contributed to the elimination of major diseases in the developed world. Fluoroquinolones, sulfonamides, aminoglycosides, tetracyclines and beta-lactams are just a few of the various available antimicrobial classes with the latter being one of the most important in both human and veterinary medicine [11, 55, 56]. In 2013, 473 tons of penicillins, which were attributed to veterinarians, made up the largest group of a total of 1.457 tons of antimicrobials in Germany. Cephalosporins of the third- and fourth-generation accounted for almost four tons [57]. Antimicrobial use in the human sector in Germany is estimated to be about 800 tons each year. Approximately one third of all these prescribed antimicrobials are beta-lactams [11, 58]. The era of beta-lactams began in 1928, with the discovery of penicillin by Alexander Fleming [59, 60]. In addition to penicillin, the cephalosporins, carbapenems, monobactams and beta-lactamase inhibitors (e.g. clavulanic acid) belong to this antibacterial class [59]. Most of them are used in humans and animals, however, carbapenems are -due to their broad spectrum

activity against Gram-positive and Gram-negative- mostly considered “anti-infectives of last resort” in humans with severe diseases [61]. Meanwhile, resistances do not only occur against first-generation beta-lactams like penicillin, but also against third- and fourth-generation cephalosporins and carbapenems [61-63].

The beta-lactams’ common feature is a cyclic amid, which harbors two carbon molecules in addition to the carbonyl moiety and nitrogen in the hetero ring system [59]. They form covalent bonds with bacterial penicillin-binding-proteins (PBPs) and affect the bacterial cell wall by blocking the peptidoglycan synthesis [64], thereby disrupting the murein layer formation [54] and thus acting bactericidal on proliferating bugs. Differences in drug effectiveness between the different groups of beta-lactams depend on their PBPs affinity, degradability through beta-lactamase enzymes and capacity to penetrate the outer bacterial membrane [65].

2.1.2 Beta-lactamases

The main resistance mechanism of Gram-negative bacteria against beta-lactam antimicrobials is the enzymatic deactivation through beta-lactamases [66]. A reduced bonding capacity of beta-lactams to PBPs by mutations and/or decreased expression of these proteins [67, 68], an enhanced substance removal out of the cells via efflux systems [69, 70] and a decreased uptake of the antimicrobial due to cell membrane transformation play a minor role as well [71].

Beta-lactamases are naturally occurring proteins in Gram-negative and Gram-positive bacteria, which hydrolyze the cyclic amid ring system of beta-lactam antimicrobials and subsequently destroy their activity [51]. Except for a few, many bacteria are able to synthesize at least one of the over 500 described beta-lactamases (www.lahey.org). According to today’s commonly used Ambler-scheme [72] (Tab. 1), enzymes are classified molecularly depending on their amino acid sequence: into metallo-enzymes with a zinc atom and those possessing serine in their active center. The serine beta-lactamases are assigned to Ambler-classes A, C and D and the metallo beta-lactamases to class B (Tab. 1).

Table 1: Beta-lactamases classification based on the Ambler-scheme [72] differentiated in serine beta-lactamases (Ambler-classes A, C and D) and metallo beta-lactamases (Ambler-class B). Their designation (e.g. ESBLs and Carbapenemases), enzyme examples (e.g. CTX-M, TEM, KPC and OXA) and their phenotypic resistance (e.g. towards penicillins and cephalosporins) are indicated in the table.

	Serine beta-lactamases					**Metallo beta-lactamases**
Ambler-class	**Class A**		**Class C**	**Class D**		**Class B**
Designation	(Extended-spectrum) beta-lactamases	Carbapenemases	Ampicillinases	Oxacillinases		Metallo beta-lactamases
Example enzymes	CTX-M, TEM, SHV	KPC, GES	AmpC, CMY, MOX, DHA	OXA-type ESBLs	OXA-type carbapenemases	VIM, NDM, IMP
Example phenotypic resistance	penicillins cephalosporins	penicillins cephalosporins carbapenems	penicillins cefotaxime	oxacillin cefotaxime	penicillins cephalosporins carbapenems	penicillins cephalosporins carbapenems

Bush *et al.* [73] classified the beta-lactamases based upon their functional groups. Group 1 contains cephalosporinases of Ambler-class C, which are hardly inhibited by beta-lactamase-inhibitors (e.g. clavulanic acid). To group 2 belong enzymes of Ambler-classes A and D, which are mostly inhibited through beta-lactamase-inhibitors. Ambler-class B enzymes belong to group 3, which are hardly inhibited by beta-lactamase-inhibitors, however, it is possible through Ethylenediaminetetraacetate (EDTA) and 4-Chloromercuribenzoic acid (pCMB). Finally, the penicillinases, which cannot be inhibited by clavulanic acid, are members of group 4 (according to the old system).

2.1.3 Extended-spectrum beta-lactamases (ESBLs)

Newer beta-lactamases are ESBLs. The term extended-spectrum describes the ability of these beta-lactamases to hydrolyze a broader spectrum of beta-lactam antimicrobials than their precursors, from which they differ in several substituted amino acids [74]. They are secondarily acquired beta-lactamases of Ambler-class A or D, which were first described in a human isolate in 1983 [75]. By definition, they are able to hydrolyze oxyimino (third-generation) cephalosporins (e.g. cefotaxime and ceftazidime). They have an active center with

serine and are inhibited by classical beta-lactamase inhibitors like clavulanic acid and sulbactam [66, 73]. ESBLs developed from primal beta-lactamases through mutations in the respective beta-lactamase (*bla*)-genes, which led to a protein structure transformation and subsequent affinity modification [74]. ESBL-producing bacteria are usually susceptible to cephamycins (e.g. cefoxitin) and carbapenems [62, 76]. In addition to ESBLs, plasmid-encoded AmpC beta-lactamases and carbapenemases belong to the newer beta-lactamases [77].

A worldwide dissemination of ESBL-producing Enterobacteriaceae, especially *E. coli* and *Klebsiella* spp., can be observed since the late 1990s [78]. The so-called early ESBLs are mainly enzymes of types TEM and SHV [77], increasingly replaced in their detection rate by CTX-M-enzymes [79]. ESBL-genes are mostly plasmid-encoded and thus easily transmitted between different bacteria via conjugation [66]. Often these plasmids not only contain ESBL-resistance genes but also those effective against other antimicrobial classes, including aminoglycosides, tetracyclines, fluoroquinolones and sulfonamides [77, 80, 81].

TEM

TEM-1 was first described in the early 1960s [82] and termed for a Greek patient named Temoneira, from whose blood sample it was isolated [83]. This beta-lactamase type has since disseminated worldwide and is able to hydrolyze penicillins and first-generation cephalosporins but no oxyimino (third-generation) cephalosporins [76]. TEM-2 differs from TEM-1 by one amino acid, which does not affect its effectivity towards beta-lactams [84]. Thus, TEM-1, TEM-2 and also TEM-13 do not belong to ESBLs [84, 85]. In 1988, Sougakoff *et al.* [86] described the enzymes TEM-3 through TEM-7, which also enable resistance against third-generation cephalosporins. Since then many extended-spectrum TEM-variants are described to be effective towards these antibiotics.

SHV

SHV means sulfhydryl variable. SHV-1 is the precursor for all SHV-derivatives and one assumes that the gene for this enzyme originated from *Klebsiella* spp. core genomes, to be then gathered by plasmids and transferred to other Enterobacteriaceae [76]. SHV-1 confers resistance against most penicillins but not against oxyimino cephalosporins [66]. In 1983, the plasmid-encoded SHV-2 was discovered, which also transmits resistances against newer-generation cephalosporins. It was one of the first discovered ESBL-enzymes [75]. Nowadays,

there are at least 127 SHV-beta-lactamases, whereat the majority belongs to ESBL-enzymes (www.lahey.org).

CTX-M

The name CTX derives from the enzyme's ability to hydrolyze cefotaxime [87, 88]. At present, CTX-M is the most common and still increasing beta-lactamase-type in human isolates [89], isolates from animals [32] and the environment [16]. Looking at the distribution of various CTX-M-types globally (Europe, Asia, America) as well as in a host context (humans, companion animals and livestock), CTX-M-1, CTX-M-14, and CTX-M-15 predominate, whereas CTX-M-1 occurs most frequently in animals from Europe. In humans, CTX-M-14 and CTX-M-15 are most common [32]. CTX-M was first described in Japan in 1988, then called FEC (Fujisawa *E. coli*) [90] and, shortly after, Bauernfeind *et al.* [91] detected a broad spectrum beta-lactamase (CTX-M-1) produced by an *E. coli* strain in Germany, which was resistant against cefotaxime and neither belonged to TEM nor SHV. A rapid dissemination of CTX-M-enzymes in various Enterobacteriaceae species was then documented, especially in South America and Europe [79, 92]. Analyses showed less than 39 % similarity between CTX-M and SHV-1 or TEM [93]. Presumably, CTX-M-enzymes descended from chromosomally encoded beta-lactamases from *Klyuvera* spp. [94, 95]. There are five main CTX-M groups, in which members of one group have to be >94 % identical and members of different groups exhibit an identity of <90 % [79].

OXA

This beta-lactamase type belongs to Ambler-class D and functional group 2d and shows a high hydrolytic effect against oxacillin and cloxacillin with an unlikely simultaneous inhibition through clavulanic acid [73]. OXA are often detected in *Pseudomonas aeruginosa* [96] but also in other Gram-negative bacteria, including *E. coli* [85]. These enzymes usually show a higher phenotypic conformity than being genotypically identical [62]. Most OXA do not belong to ESBLs, with the exception of OXA-10, OXA-14, among others [85, 97-99]. The most prominent is OXA-48, which is a carbapenemase [100, 101].

2.2 *Escherichia coli*

- ESBL-PRODUCING *E. COLI* EXEMPLIFY THE WORLDWIDE THREAT OF AMR

E. coli is one of the key organisms in the field of acquired antimicrobial resistance found in human and veterinary medicine but also in extra-clinical settings [11]. *E. coli* bacteria are sometimes resistant against a broad range of different antimicrobial classes, including beta-lactams and fluoroquinolones, which results in a multi-resistant phenotype and limitations in antimicrobial therapies [80, 81, 102]. ESBL-producing representatives of these Gram-negative, facultative anaerobic rod-shaped bacteria form one group of "superbugs" ("colloquial reference to a bacterium that carries resistance genes to many antibiotics or to a bacterium or virus of greatly enhanced virulence" [103]) together with methicillin-resistant *Staphylococcus aureus* (MRSA) [104], vancomycin-/multi-drug-resistant *Enterococcus* (VRE) [105], multi-drug-resistant *Pseudomonas aeruginosa* [106], *Clostridium difficile* [107], ESBL-producing *Klebsiella pneumoniae* [108], carbapenem-resistant Enterobacteriaceae (CRE) [109] and *Acinetobacter baumanii* [110]. *E. coli* belong to the family of Enterobacteriaceae and were discovered by Theodor Escherich in 1885. Apathogenic (commensal) are distinguished from intestinal pathogenic (InPEC) and extra-intestinal pathogenic *E. coli* (ExPEC) [77, 111, 112]. InPEC and ExPEC consist of various pathotypes [112], which are displayed in Figure 2.

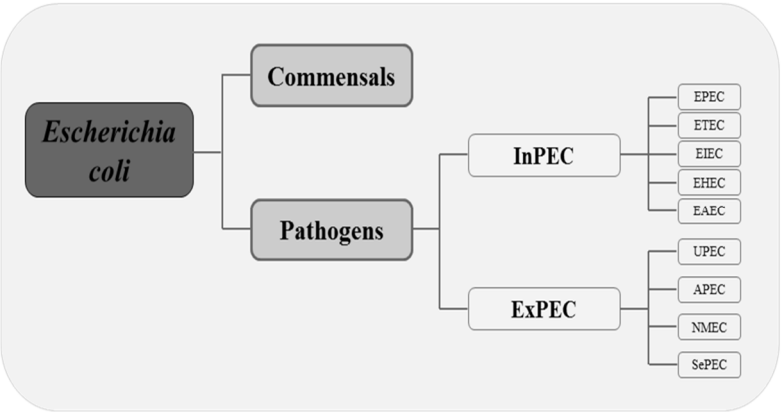


Figure 2: *E. coli* subtypes distinguished into commensals and pathogens. Abbreviations: InPEC= Intestinal pathogenic *E. coli* (consisting of EPEC: Enteropathogenic *E. coli*, ETEC: Enterotoxigenic *E. coli*, EIEC: Enteroinvasive *E. coli*, EHEC: Enterohemorrhagic *E. coli*, EAEC: Enteroaggregative *E. coli*), ExPEC= Extra-intestinal pathogenic *E. coli* (consisting of UPEC: Uropathogenic *E. coli*, APEC: Avian pathogenic *E. coli*, NMEC: Neonatal meningitis *E. coli*, SePEC: Septicemic *E. coli*).

Commensal *E. coli* are components of the physiological microbiota in humans and warm-blooded animals and birds [54, 113] and are found globally in different habitats and hosts, underlining their significance as indicator strains in water [114] and food [115] safety. They are one of the first colonizers in a newborn's gut, transferred through the mother already during birth [65]. They provide the host, for instance, with vitamin K2 and may prevent intestinal colonization with pathogens [116, 117].

As mentioned above, *E. coli* is also a ubiquitous pathogen and a frequent cause of urinary tract infections, bacteremia, soft tissue infections and diarrhea [112], and other diseases in humans [118] and animals [32]. Intestinal and extra-intestinal infections lead to enhanced mortality, morbidity and to extensive costs in public health [77]. As an example in veterinary medicine, poultry infections caused by avian pathogenic *E. coli* result in considerable economic problems due to enhanced damage, reduced hatchability, decreased production output and carcass contamination [119].

The strains used in this thesis mainly represent ExPEC and commensal *E. coli*, as ESBL-production seems to be associated with these types [11], however, InPEC isolates have been reported to be ESBL-producers as well, for instance during the EHEC-outbreak in Germany in 2011 [120].

2.3. ESBL-producing *E. coli* in different habitats and hosts

- ESBL-PRODUCING *E. COLI* OCCUR IN CLINICAL AND EXTRA-CLINICAL SETTINGS

2.3.1 The occurrence of ESBL-producing *E. coli* in humans and domesticated animals

Since resistance mechanisms to third-generation cephalosporins were first detected in a clinical human *Klebsiella* isolate in 1983 [75] (Fig. 3), and then in a nosocomial outbreak in 1988 [121], a rapid, global dissemination of ESBL-producing *E. coli* in human clinical environments [18, 21, 122] and the community [8, 78] has been documented. The prevalence of ESBL-producing Enterobacteriaceae varies globally; in 2010, it was highest in *E. coli* in Asia/Pacific (28 %), followed by Latin America (23.0 %), Europe (19 %) and North America (7 %) [123]. While comprehensive studies on the rates of ESBL-producers in Africa are largely unavailable, numbers approaching 70 % have been detected in Egypt [124].

One of the first clinical ESBL-producing isolates discovered in animals was a SHV-12-producing *E. coli* strain from a dog with recurring urinary tract infections in Spain [6] (Fig. 3). In 2003, ESBL-producing *E. coli* have been isolated from feces of healthy chickens in Spain [125], from the feces of healthy broilers in Japan [126] and cattle, pigs and a pigeon in Hong Kong [127].

Currently the most common ESBL-encoding genes in both livestock and companion animals are CTX-Ms (*bla*$_{\text{CTX-M-1, 15, 14, 9}}$), followed by *bla*$_{\text{TEM-52}}$, *bla*$_{\text{SHV-12}}$ and other TEM and SHV types [32]. CTX-M-1, for example, is the most frequent enzyme type in farm animals in Europe. Interestingly, this does not apply for humans, in whom CTX-M-14 and CTX-M-15 are the most common [32].

2.3.2 The occurrence of ESBL-producing *E. coli* in wildlife and the environment

The presence of AMR bacteria and genes in the environment is explained by a mix of naturally occurring resistances [49] and those from animal and human origin [16]. This is due to three main mechanisms: horizontal gene transfer, chromosomal mutation and recombination, and selective pressures owed to other compounds, for example heavy metals [17]. Environmental hotspots for AMR bacteria and genes include wastewater treatment facilities, aquaculture, pharmaceutical manufacturing and animal husbandry effluents [17].

Clinically relevant ESBL-producing *E. coli* demonstrate the ubiquity of AMR bacteria. Since 2006, multiple studies have reported on the presence of these bacteria in wildlife [13, 15, 37, 38, 128-135] (Fig. 3), often associated with human activities and influences generally [136, 137], through fecal water contamination in particular [16]. ESBL-producing *E. coli* also appear in remote areas [12, 134, 138], which might be due to other factors, such as their dissemination via wild bird migration. The extent of commensal *E. coli* intestinal colonization varies among different bird and mammal species [40, 139] and is most frequently detected in omnivorous representatives of these classes [139]. In the broad class of birds, waterfowl and birds of prey groups seem to play an important role regarding ESBL-carriage [15, 37, 38, 129, 131, 133-135]. The occurrence of ESBL-producing *E. coli* in waterfowl might be likely associated with fecal water contamination and in birds of prey with infected prey animals and their position in the food chain [14]. Besides birds, rodents are important hosts for ESBL-producing *E. coli*, whereat Norway and Black rats predominate [13, 140]. Their synanthropic way of life leads to a putatively easy transmission of feces to other animals and humans [14].

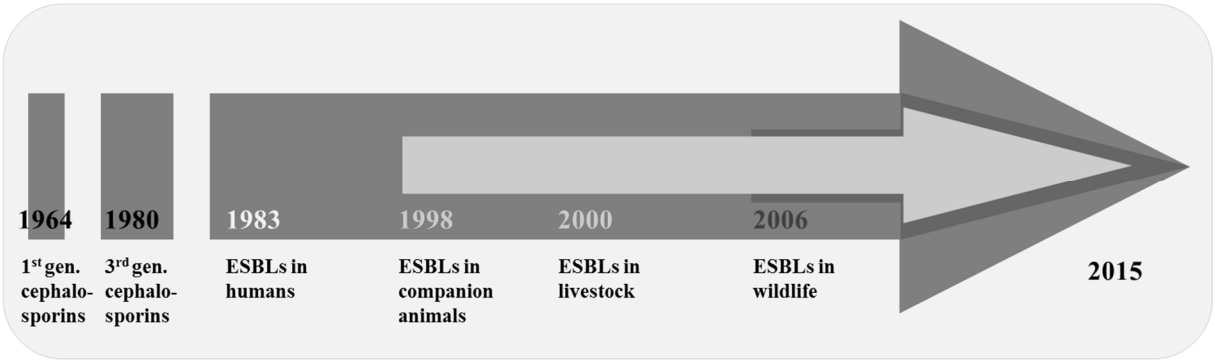
Other wildlife animals, which sporadically carry ESBL-producing bacteria include wild boars [132], deer, foxes [130], fishes [128], insects [141] and even Spanish slugs [142]. The most dominant ESBL-gene family detected in wildlife is *bla*$_{\text{CTX-M}}$, with *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CTX-M-14}}$ and others as the most frequent types [14, 143].

Some studies report on the occurrence of ESBL-producing *E. coli* in aquatic ecosystems including wastewater and sewage sludge [35, 144, 145] as well as river [146], pond [15] and lake water [36]. Surface waters of populated regions almost always contain antimicrobials constituting selective pressures on the bacteria they accommodate [147]. Other factors, however, including contaminants and bacterial adaptation, may also contribute to the dissemination of antimicrobial resistant populations in extra-clinical settings [17].

While data on environmental ESBL-producing *E. coli* are mainly limited to studies addressing the habitats and hosts mentioned above, little information is available concerning these bugs in dog feces from environmental settings. Publication I in this thesis is one of the first to report on these antimicrobial resistant bacteria in dog feces collected from the environment [41, 148]. Other multi-resistant bacteria including *Enterococci* though, have been described from dog feces collected on urban streets in Italy in 2013 [149]. The occurrence of ESBL-producing *E. coli* in environmental dog feces is not surprising as dogs are often carriers of these germs, be it in clinical [30, 150] or asymptomatic [34, 151] colonization contexts. Fecal droppings subsequently lead to environmental pollution with resistant bacteria, embodied not only as part of the dog fecal matters but also, for instance, in related aquatic ecosystems [152, 153].

In general, the exact contribution of resistant bacteria and genes from the environment to the spread of antimicrobial resistance among clinically relevant bacteria is not well understood. Global characterization and quantification approaches of these determinants are necessary to elucidate fully the environment's role in dissemination scenarios [17].

Figure 3: Time arrow with first-generation cephalosporins brought onto market in 1964 [154], third-generation cephalosporins in 1980 [155, 156] and the subsequent occurrence of ESBLs in different habitats and hosts (humans [75], companion animals [6], livestock [157] and wildlife [130]).



2.4 Zoonotic potential of ESBL-producing *E. coli* in a One Health perspective

- ESBL-PRODUCING *E. COLI* ARE ZOONOTIC AGENTS AND TRANSMITTED ACROSS DIFFERENT HOSTS AND SETTINGS HIGHLIGHTING THEIR STUDY POTENTIAL IN THE CONTEXT OF ONE HEALTH

One Health describes the worldwide initiatives at the nexus of human, animal and environmental health. It is crucial for the future prevention and control of infectious diseases, with AMR bacteria holding a key role in this combat [1]. Most infectious diseases in humans are zoonoses, defined as the possibility of natural transmission of the infectious agent between animals and humans. This dissemination is not unidirectional, as humans' omnipresent interventions also often lead to morbid animals and habitats [1].

E. coli thrive in the gut of humans and animals, but also play an important role in the environment. Their persistence and AMR ability outside the (human and animal) organism present an opportunity to study putative transmission scenarios among humans, animals and the environment, and to elucidate their role as a source of non-point and re-infections. Figure 4 displays these possible transmission ways in a simplified manner.

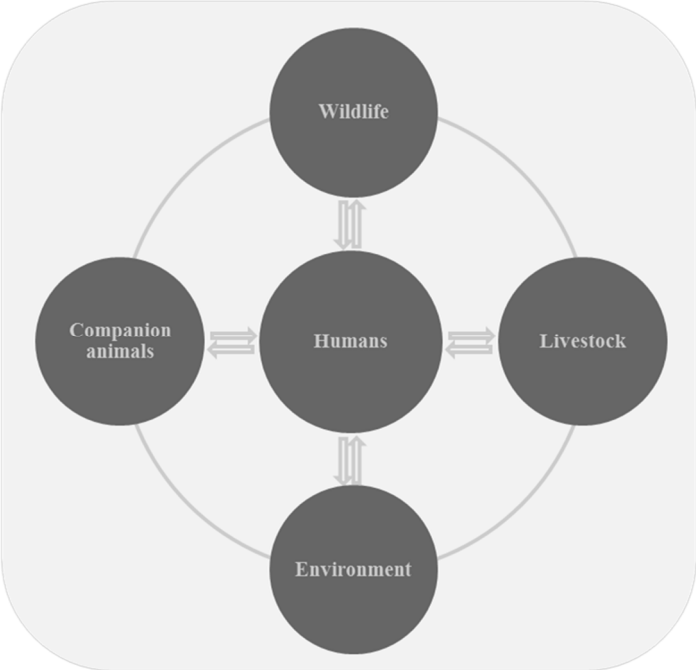


Figure 4: Possible transmission ways of ESBL-producing *E. coli* between different habitats and hosts. (Companion animals: pets that provide health benefits to a person [here: dogs, cats, horses etc.]*, livestock: agricultural animals/ animals kept for profit [here: poultry, cattle, pigs etc.]**, wildlife: animals that live unrestrained in a natural environment [here: wild birds, wild rodents etc.]**, environment: factors that influence the life and survival of a person [here: soil, water, air etc.]*, [*: Mosby, Inc., *Mosby's Medical Dictionary* 2009, Elsevier: New York (USA) **: Studdert, P.V., *et al.*, *Saunders Comprehensive Veterinary Dictionary*, 2007, Elsevier: New York (USA)]).

The increasing occurrence of ESBL-producers in livestock (**) [103] and companion animals [26, 143] (*) [158], led to the assumption that they are relevant infection sources for the dissemination of multi-resistant bacteria for the environment and to humans. Since the natural habitat of *E. coli* is the gut, a spread of these bacteria into the environment via manure is nearly inevitable [159], subsequently contaminating vegetables and crops [160]. Companion animals are particularly interesting in the context of increasingly close contact between pet and owner [161, 162]. In developed countries especially, pets are often members of the family, constituting a perfect setting to exchange bacteria with their human companions [163-165]. The omnipresence of dog feces in outdoor settings leads to environmental pollution with zoonotic and resistant bacteria in various habitats [166], extending this infection scenario to other humans and animals beyond the family [167]; similar scenarios also apply to avian residues [15, 131].

The contamination of the environment with AMR bacteria in close proximity to both human and veterinary hospitals, for instance via wastewater effluents, is crucial in the dissemination of resistant bacteria as well [168-170]. Wastewater treatment does often not sufficiently eliminate antimicrobial resistant bacteria, which then may re-enter farmed land and subsequently the food chain via treated sewage [171].

Clonal and phylogenetic relationships as well as transmission scenarios of multi-resistant bacteria among humans, animals and the environment are reinforced by several multi-locus

sequence typing (MLST) studies [12, 14, 29, 38, 172-179], which show that ESBL-producing *E. coli* in these habitats possess the same sequence types (STs). This indicates an interspecies transmission of phylogenetically and clonally related strains.

To examine AMR, zoonotic bacteria in a One Health approach, it is necessary to characterize the same pathogen molecularly from clinical contexts, but also in its related extra-clinical environments.

2.5 Characterization of *E. coli*

- THE CHARACTERIZATION OF ESBL-PRODUCING *E. COLI* IS INEVITABLE TO STUDY THEIR MOLECULAR, PHYLOGENETIC AND CLONAL RELATIONSHIPS AND TO INVESTIGATE TRANSMISSION SCENARIOS EXEMPLARY FOR THE GLOBAL THREAT OF AMR

E. coli may be characterized by various classification and typing methods, whereat Achtman [180] defines classification as a top-down approach to distinguish within bacterial species needed for long-term epidemiology and the typing methodologies as bottom-up approaches to differentiate among bacterial isolates as a part of, together with the classification techniques, short-term epidemiology (Fig. 5). Risk assessment (e.g. zoonotic and virulence potential) is regarded as being only possible via typing.

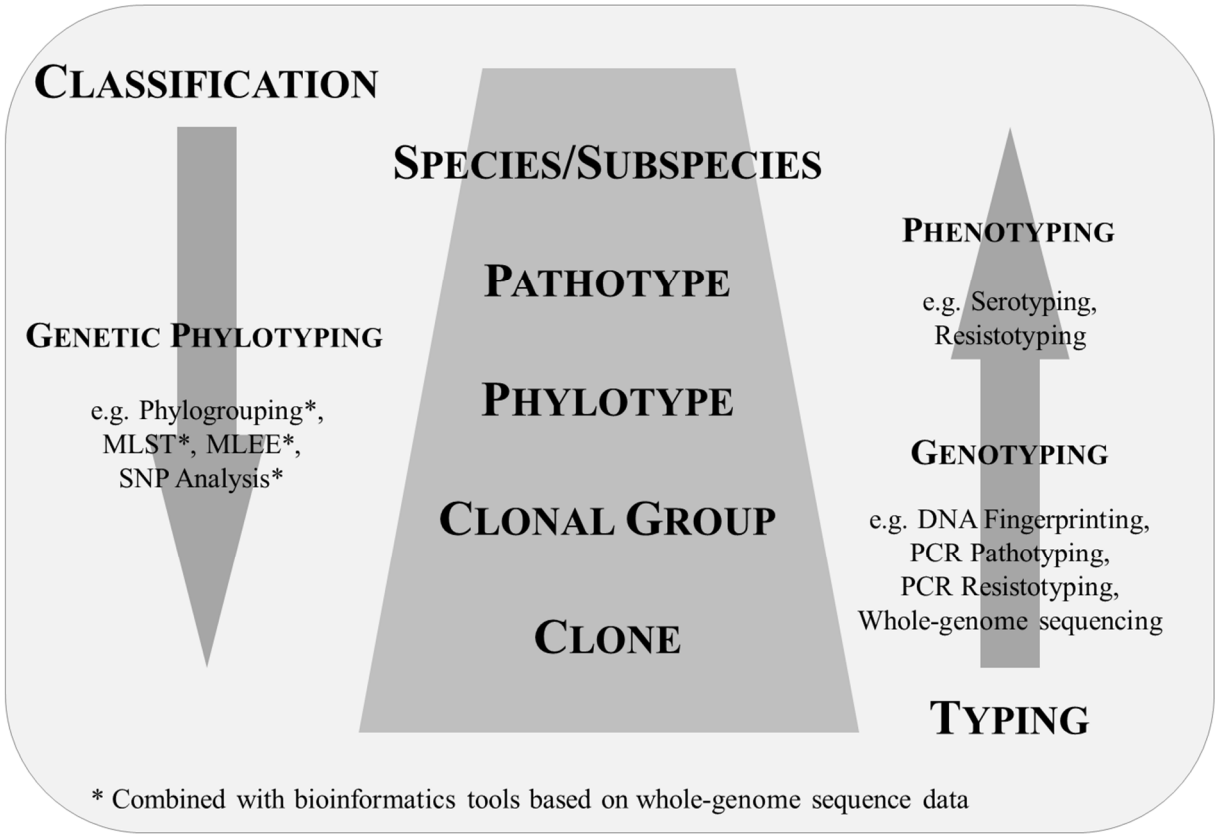
Bacterial species identification is historically based on colony morphology and growth, Gram staining, sugar utilization and biochemistry, and was then completed with MALDI-TOF [181] or 16S rRNA sequencing [182]. To elucidate transmission scenarios of AMR *E. coli* among humans, animals and the environment, however, molecular (epidemiological) methodologies including the examination of population genetics and phylogenetics play a key role.

Bacteria are defined by both phenotypic and genotypic characteristics [182]. Traditional typing methods for bacteria focusing on their phenotype, such as the serotype and resistance phenotype, have been employed for many years [183] and are relevant in vaccine development or disease treatment. They are, however, not ideal for modern epidemiology as the phenotype does not necessarily match the genotype. Genetically distant strains may have the same serotype and vice versa: closely related strains may exhibit different serotypes [183]. In addition serological markers and resistance factors are frequently acquired by horizontal gene transfer, are thus not selectively neutral and easily transmittable; also between phylogenetically distant strains [180]. Besides being fast, easy and reliable, classification

methodologies require selectively neutral markers, distributed over the chromosomal content, and a reflection of the bacterial population including population genetics and phylogenetics [180, 184, 185].

This literature survey describes *E. coli* characterization tools most relevant for the thesis only. In general, when choosing an appropriate method for particular (epidemiological) investigations, several factors should be considered: the time until receiving results, the ability to differentiate non-clonal isolates, the reproducibility of results, the interpretability of results, comparability between different laboratories, and available resources and expense factors [186].

Figure 5: Characterization possibilities of *E. coli* distinguished into classification and typing methods. Abbreviations: MLST=Multi-locus sequence typing, MLEE=Multi-locus enzyme electrophoresis, SNP=Single-nucleotide polymorphism. *=combined with bioinformatics tools based on whole-genome sequence data.



2.5.1 Multi-locus sequence typing (MLST)

Molecular characterization methods use sequence variability due to mutation and/or recombination to analyze bacterial relationships [186]. One of them is MLST, where *E. coli* are characterized by the assignation to STs [187]. MLST uses nucleotide fragments of, in general, seven different gene loci (for *E. coli* usually: *adk*, *fumC*, *purA*, *recA*, *gyrB*, *icd* and *mdh*) of the conserved chromosomal genome ("housekeeping genes") since these are most likely selectively neutral [183]. Although MLST primarily represents a typing method, it can also be used to classify bacteria by using bioinformatics tools. Following PCR-amplification, the fragments are sequenced and characterized based on DNA-sequence variations; every unique allele combination results in a particular ST. Point mutations and horizontal gene acquisition are equally weighted as the number of differing nucleotides between different alleles is ignored [180]. Related STs are grouped to a sequence type complex [188]; the at least three complex members do not differ in more than two of the seven loci compared to their complex neighbor. MLST reveals a high resolution of *E. coli* population genetic structures, in addition constituting the advantage of a simple electronic data exchange and reproducibility between independent laboratories (www.mlst.net), with the pre-requisite of high quality sequencing [186]. In clinical ESBL-producing *E. coli*, the discriminatory index for MLST typing was higher than the one for pulsed field gel-electrophoresis (PFGE) (see below) [189], even though other authors report the opposite for *E. coli* O157:H7 [190].

2.5.2 Phylogenetic grouping and the EcoR strain collection

E. coli are differentiated into four phylogenetic groups: A, B1, B2 and, D. ExPEC mainly belong to group B2 and, to a lesser extent, group D [191-193]. Commensal subtypes are mostly found in groups A and B1 [191, 192, 194]. The group affiliation is originally based on multi-locus enzyme electrophoresis (MLEE). Ochman and Selander [195] developed a standardized reference collection consisting of 72 *E. coli* isolates from different origins including pathogens and commensals. The collection was regarded completely representative for the variety of *E. coli* and subsequently designated EcoR (*E. coli* reference). It described a clonal population structure, assigned to four phylogenetic groups A, B1, B2 and D and a less important group E. Since its first introduction, the EcoR collection has been a main subject to study *E. coli* diversity with many studies supporting the reliable grouping into phylogenetic groups. Different methods included restriction fragment length polymorphism (RFLP) and

randomly amplified polymorphic DNA (RAPD) [196]. In 2000, a rapid and simple phylogrouping triplex PCR was introduced by Clermont and colleagues [197] allowing the assignment of *E. coli* strains to one of the four main phylogenetic groups using the combination of two genes (*chuA* and *yjaA*) and a DNA fragment (TSPE4) [198].

Later, in 2006, 462 *E. coli* isolates of different origins, including the 72 EcoR strains, were multi-locus sequence typed by Wirth *et al.* [199]. These strains reflected a by far higher genetic variability than previously assumed. Based on their analyses, these authors discussed that the population development most likely occurred in the last five millions of years after a bottleneck event, where the population size rose by 50 times. STRUCTURE, which is based on a Bayesian mathematically model and which analyses the concatenated sequences of seven "housekeeping genes" obtained with MLST (software: STRUCTURE 2.3.X used for MLST) revealed four main phylogenetic groups within *E. coli* strains and divided the EcoR-strains into fractions. The isolates accorded to the original groups A, B1, B2 and D. Two third of the 462 examined strains belonged to these four groups, whereas one third was assigned to two hybrid groups (AxB1 and ABD) reflecting the emergence of strains of different sources with recombination as the driving force for this phenomenon. Thus for the first time it was shown that sequencing a rather small part of the genome (roughly 3500 bp) was adequate to not only reproduce the population structure as defined by MLEE of 20 proteins, but even provided a higher resolution.

2.5.3 DNA Fingerprinting

DNA Fingerprinting is useful to subtype various bacterial species. It includes macrorestriction analysis with subsequent PFGE [200] and ribotyping [201, 202] and was initially developed to detect clonally related strains and to investigate outbreaks [184, 203], for example of *E. coli* O157:H7 [203]. To apply PFGE, bacteria are lysed and the chromosomal content is digested with endonucleases that cleave the genome at infrequently occurring restriction sites [204]. The resulting DNA fragments may then be separated in a gel, which in contrast to the standard gel electrophoresis protocol varies the orientation of the electric field in the gel during the run ("pulse"). This by that time new approach allowed the separation of DNA fragments up to 1 Mb [205]. The separated fragments are visualized on the gel as bands, generating a specific PFGE pattern. Differences in these patterns are used to compare bacterial isolates on a genetic level [186], where those differing by a single genetic event reflected by two to three fragments are considered closely related [186, 200]. PFGE addresses

most of the investigated genome (>90 %), however, it may be error-prone in terms of the differentiation of bands with nearly identical sizes [184]. Until recently, it was often described the "gold standard" of molecular clonal typing methods in *E. coli* and other Enterobacteria and exhibits advantages compared to PCR-based methods due to the consideration of the whole genome. Standardized PFGE protocols are used to achieve high reproducibility between different laboratories. On the other hand, this method is time-consuming and, the discriminatory power may be decreased if a genetic change does not affect the electrophoretic ability of the digested fragment [186]. In general, systems based on the differentiation of bands are prone to errors due to a challenging size assignment.

2.5.4 High-throughput sequencing (HTS) applications

For a long time, MLST and macrorestriction analyses were regarded the reference standard for bacterial characterization but they reveal a lack in the discriminatory power for isolates evolving from one single clone [182, 206, 207]. Whole-genome sequence data enable the more precise determination of genomic contents of bacteria and the identification of single genomic differences between isolates, representing an important tool for epidemiological and outbreak investigations [184, 208]. Since 2005, (ultra-) high-throughput (or next-generation) sequencing applications mostly replaced Sanger sequencing [209-211] resulting in an enhanced simplicity with concurrently decreased costs and run times [212]. HTS offers possibilities for the fast analysis of pathogens decreasing the normally needed various steps for a detailed molecular characterization [182]. Next-generation sequencing (NGS) platforms are roughly divided into two groups, which is dependent on the used template: template amplification technologies with a usually three step workflow of library preparation, amplification and sequencing; and single molecule sequencing without amplification [213]. The platforms and different machines also differ in resulting read lengths, throughput, run time, sequencing costs and, error rate. Further, machines may be divided into high-end instruments (e.g. PacBio RS (from Pacific Biosciences), Illumina HiSeq, and the Life Sciences 454 GS FLX+) with massive throughputs, due to high costs probably convenient only for core facilities and, bench-top instruments (e.g. Illumina MiSeq and the Life Sciences 454 GS Junior), which are not as expensive but adequate for standard laboratories [213]. Whole-genome sequence data are the base for multiple analyses and tools, for example core and accessory genome comparisons including single-nucleotide polymorphism (SNP) approaches and the analysis of mobile genetic elements and the presence of resistance factors

(resistome) and toxins (toxome) [182]. SNP analysis uses nucleotide variations following a mutation event to differentiate closely related bacterial isolates and to elucidate the evolutionary history of particular microorganisms [214]. Bertelli and Greub state, that it is even possible, when the rate of SNPs is high enough, to trace transmission routes among independent hospitals and patients [182]. The analysis of whole-genome sequence data helps tracking the dissemination of antimicrobial resistant bacteria globally and gives insights into phylogenetic population structures, transmission pathways and bacterial evolution, also of ESBL-producing *E. coli.* According to Le and Diep [215] it "provides the ultimate in pathogen strain resolution". Whole-genome approaches should be considered the future gold standard for phylogenetics and typing in *E. coli*, especially because analyses using limited datasets like MLST are more easily affected by recombination events [183]. This does not apply for whole-genomes; at least at a level to disrupt their phylogenetic characteristics [216]. At the moment, however, whole-genome assembly and information delivery is mostly reserved for highly skilled people like bioinformaticians. In addition, making the massive amount of data available for the whole scientific community is challenging. This calls for standard sequencing and analysis procedures, fully automated and easy bioinformatics pipelines as well as collaborations in biology, medicine, and bioinformatics [182, 215].

3. PUBLICATIONS

3.1 Publication I

Putative connection between zoonotic multiresistant extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in dog feces from a veterinary campus and clinical isolates from dogs

DOI: 10.3402/iee.v5.25334

- Published in the peer-reviewed journal Infection Ecology and Epidemiology
- Received: 01.07.2014
- Accepted: 03.01.2015
- Published: 04.02.2015

3.2 Publication II

Clonal spread and interspecies transmission of clinically relevant ESBL-producing *Escherichia coli* of ST410 – another successful pandemic clone?

DOI: 10.1093/femsec/fiv155

- Published in the peer-reviewed journal FEMS Microbiology Ecology
- Received: 01.09.2015
- Accepted: 26.11.2015
- Published online: 10.12.2015

Please purchase this part online.

INFECTION ECOLOGY & EPIDEMIOLOGY
THE ONE HEALTH JOURNAL

COACTION PUBLISHING

ORIGINAL RESEARCH ARTICLE

Putative connection between zoonotic multiresistant extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in dog feces from a veterinary campus and clinical isolates from dogs

Katharina Schaufler, PhD Student[1]*, Astrid Bethe, PhD[1], Antina Lübke-Becker, PhD[1], Christa Ewers, PhD[2], Barbara Kohn, PhD[3], Lothar H. Wieler, PhD[1] and Sebastian Guenther, PhD[1]

[1]Centre for Infection Medicine, Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany; [2]Institute of Hygiene and Infectious Diseases of Animals, Justus-Liebig Universität Giessen, Giessen, Germany; [3]Clinic of Small Animals, Faculty of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

Introduction: To contribute to the understanding of multiresistant bacteria, a 'One Health' approach in estimating the rate of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and getting insights into the transmission from clinical settings to the surrounding environment was employed by collecting fecal samples of dogs in a public area. Isolates were compared to those from samples of diseased dogs from a nearby small-animal clinic.

Materials and methods: One hundred fecal samples of dogs were collected on a single day in the public area of a veterinary faculty with a small-animal clinic and adjacent residential neighborhoods. All identified ESBL-producing strains were isolated by selective plating, genotypically analyzed by DNA microarray, polymerase chain reaction, sequence analysis, and pulsed-field gel electrophoresis and compared to 11 clinical ESBL/AmpC-producing *E. coli* isolated from diseased dogs treated in the small-animal clinic 2 months before and 2 months following the environmental sampling collection.

Results and discussion: Fourteen percent (14/100) of the extra-clinical samples harbored phenotypic ESBL/putative AmpC-producing *E. coli* with additional resistances against other antimicrobials. One ESBL-strain displayed an identical macrorestriction pattern to one clinical, another one to three clinical clonal ESBL-producing strains. The genotypic ESBL-determinants ($bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$) and detection rates (10%) in dog feces collected outside of the small-animal clinic are comparable to the rates and ESBL-types in the healthy human population in Germany and to clinical and non-clinical samples of humans and companion animals in Europe. The occurrence of identical strains detected both outside and inside the clinical setting suggests a connection between the small-animal clinic and the surrounding environment.

In conclusion, dog feces collected in proximity to veterinary facilities should be considered as a non-point infection source of zoonotic ESBL-producing *E. coli* for both animals and humans. The common sniffing behavior of dogs further urges hygienic measures on the part of dog-patient owners, who should be educated to remove their pet's feces immediately and effectively.

Keywords: *antimicrobial resistance*; *urban environments*; *environmental spread*; *clonality*; *shared ESBL-STs*

Responsible Editor: Elsa Jourdain, INRA, Centre de recherche de Clermont-Ferrand/Theix, Saint Genès Champanelle, France.

*Correspondence to: Katharina Schaufler, Centre for Infection Medicine, Institute of Microbiology and Epizootics, Freie Universität Berlin, Robert-von-Ostertag-Strasse 7-13, DE-14163 Berlin, Germany, Email: katharina.schaufler@fu-berlin.de

Received: 1 July 2014; Revised: 16 November 2014; Accepted: 3 January 2015; Published: 4 February 2015

Extended-spectrum beta-lactamase (ESBL) enzymes have become abundant. They are not only able to hydrolyze penicillins but also newer, third-generation cephalosporins and monobactams. Limitations in antimicrobial therapies result from a multidrug-resistant (MDR) phenotype present in these bacteria, which are often additionally resistant against fluoroquinolones, aminoglycosides, and other classes of antimicrobials (1).

Citation: Infection Ecology and Epidemiology 2015, **5**: 25334 - http://dx.doi.org/10.3402/iee.v5.25334
(page number not for citation purpose)

ESBL-producing *Escherichia coli* in humans, livestock, wildlife, and in non-clinical (2–4) and clinical isolates of companion animals originating from soft tissue, enteric, or urinary tract infections (5) are well documented. Limited data, however, are available concerning the rate of these bacteria in dog feces outside of clinical settings worldwide (6) as well as regarding a possible transmission of multiresistant bacteria from veterinary clinics into the environment. Berlin is the city in Germany with the most dogs per inhabitant (7). The dog often lives in close contact with its human companion and sometimes shares home and bed (8, 9) and routinely leaves excrement on streets and fields. It is highly interesting to what extent canine feces may carry zoonotic, multiresistant *E. coli* and its implications for transmission pathways in public health. Also, the keeping of dogs has been recently identified as a putative risk factor for ESBL secretion in owners (10). The aim of this study was to provide a 'One Health' approach in estimating the rate of ESBL-producing *E. coli* in dog feces outside of clinical settings. The samples were collected on a publicly accessible veterinary campus with small-animal clinic in Berlin to assess a possible link between the clinic and the surrounding environment, acknowledging, however, that the proof of a direct epidemiological link would be difficult to establish. A likely contamination of the environment surrounding small-animal veterinary hospitals with multiresistant bacteria was previously demonstrated by Ghosh et al. (11). In addition, samples were collected from adjacent residential neighborhoods of the veterinary faculty. This study detected high rates of ESBL-producing *E. coli* outside the clinical setting and discovered clonal strains from extra-clinical dog feces and clinical dog isolates.

Material and methods

The samples were collected on a campus of the veterinary faculty and adjacent neighborhoods in the city of Berlin (Germany) within a 24-hour period in June 2013. The area (approximately 2.5 square kilometers) was divided into five equal sized districts. Individual fecal residues, presumably from dogs, were collected using 20 swabs from each district providing 100 total fecal samples. Data collection procedures called for there to be at least 10 meters of distance between the sampling sites and to collect only fresh appearing samples to avoid sampling the same animal more than once. Third-generation cephalosporin-resistant bacteria were isolated using cefotaxime (4 μg/ml)-containing CHROMagar™ plates and were confirmed as *E. coli* via classical biochemical methods including glucose fermentation (Kligler agar), lysin-indol-motility (LIM agar), and urea fermentation (12). One putative ESBL-producing *E. coli* isolate per sample was further processed. Confirmatory testing (BD BBL® Sensi-Disc [Becton, Dickinson, Germany]) using the control strain *K. pneumoniae* ATCC®700603 analyzed the occurrence of not only ESBL-producing strains but also putative AmpC-producing strains (13). Additional resistances to ampicillin, amoxicillin–clavulanic acid, gentamicin, kanamycin, doxycycline, sulfonamide–trimethoprim, chloramphenicol, nalidixic acid (BD BBL® Sensi-Disc [Becton, Dickinson, Germany]), and enrofloxacin (Bayer, Germany) were tested using agar disc diffusion testing according to the CLSI guidelines with the control strain *E. coli* ATCC®25922 (13). Following DNA isolation, resistance genotyping of the strains was performed via DNA microarray (Alere technologies, Jena, Germany, Array strip® *E. coli*) (14). Hybridization results for $bla_{\text{CTX-M-like}}$, $bla_{\text{TEM-like}}$, $bla_{\text{OXA-like}}$, and plasmidic AmpC-genes like $bla_{\text{CMY-1}}$ were verified via polymerase chain reaction (PCR) and sequencing as described previously (15, 16). The phylogenetic group was determined using Structure 2.3.4 software based on the concatenated sequences of the seven housekeeping genes used for multilocus sequence typing MLST) (University of Chicago [http://pritch.bsd.uchicago.edu/structure.html]). MLST determination was carried out as described previously (17). Gene amplification and sequencing were performed using primers specified on the *E. coli* MLST website (University of Warwick [http://mlst.warwick.ac.uk/mlst/]). Sequences were analyzed with the software package Ridom SeqSphere 0.9.39 (Ridom website [http://www3.ridom.de/seqsphere]) and sequence types (STs) were computed automatically. Pulsed-field gel electrophoresis (PFGE) was used as recently described (15) to detect clonally related extra-clinical ESBL-/putative AmpC-producing strains, to estimate the impact of double sampling and to compare the extra-clinical samples to 11 previously confirmed ESBL/putative AmpC-producing *E. coli* isolates from diseased dogs, which were collected in the small-animal clinic 2 months before and 2 months following the extra-clinical sampling time point. This time period was chosen in order to increase the odds of finding clinical strains with similar macrorestriction patterns compared to those isolated from the extra-clinical dog feces considering the low overall detection of ESBL-producing *E. coli* in the clinic. Samples collected from diseased dogs included wound tissue, feces, and urine.

Results

In an area of approximately 2.5 square kilometers, 16 of 100 extra-clinical fecal samples revealed cefotaxime-resistant *E. coli,* 12 of which were ESBL producers and four putative AmpC producers. PFGE detected clones of two ESBL-producing *E. coli* strains (which were removed from the analysis), resulting in 10 (10%) ESBL-producing, four (4%) putative AmpC-producing, and overall 14 (14%) non-clonal strains. At least one ESBL-producing *E. coli* was found in all five districts, with no differential repartition. Thirteen strains (including ESBL- and putative AmpC-producers) were resistant to three or

Citation: Infection Ecology and Epidemiology 2015, **5**: 25334 - http://dx.doi.org/10.3402/iee.v5.25334

more than three antimicrobial classes, thus showing a multiresistant phenotype. Resistance to ampicillin (14/14), amoxicillin–clavulanic acid (10/14), gentamicin (9/14), kanamycin (12/14), doxycycline (13/14), and sulfamethoxazole–trimethoprim (12/14) was common, but chloramphenicol (4/14), nalidixic acid (7/14) and enrofloxacin-resistance (5/14) also occurred (Fig. 1a). Resistance genotyping using DNA microarray accorded mostly with the phenotypic results (see Fig. 1a for details). All ESBL-producing strains (10/10) showed positive signals for the $bla_{\text{CTX-M-group-1}}$ probe, which was confirmed by sequencing ($bla_{\text{CTX-M-1}}$ [5/10], $bla_{\text{CTX-M-15}}$ [5/10]). All putative AmpC-producing strains (4/4) were initially positive for the $bla_{\text{MOX-CMY-9}}$ probe (a class C ESBL-precursor) and additionally harbored $bla_{\text{CTX-M-1}}$, but CMY-1 could only be verified for one strain (IMT31346) with PCR. Other AmpC genes including bla_{FOX}, bla_{MOX}, and bla_{ACT} were negative (16). Analysis also detected $bla_{\text{TEM-1}}$ (7/14) and $bla_{\text{OXA-1}}$ (5/14). Non-beta-lactam resistance genes were *tetA*/*B* (13/14), *sul1*/*2* (13/14), *strA*/*B* (7/14), *catA1*/*B3*/*B8* (9/14), as well as *aadA4* (8/14), *aac(6')-II* (5/14), *dfrA7*/*17*/*19*/*21* (12/14), and *qnrS* (1/14) (Fig. 1a).

The 10 ESBL-producing strains belonged mostly to phylogenetic group B1 (7/10), three strains belonged to groups D, A, and hybrid group AxB1 (1/10 each). The putative AmpC-producers belonged to ECOR groups B1 (2/4), B2 (1/4), and D (1/4) (Fig. 1a). MLST revealed seven different STs (e.g. ST410 and ST10) among ESBL-producers and four different ones among putative AmpC-producing isolates (see Fig. 1a for details).

PFGE revealed one environmental strain (IMT31359), which showed the same macrorestriction pattern as one clinical isolate (IMT31487) and another one (IMT31356), which was identical to three clonal isolates (IMT31286, IMT31333, and IMT31711) from individual dogs treated in the small-animal clinic (Fig. 1b).

Discussion

A substantial number (14%) of the extra-clinical dog feces tested harbored ESBL/putative AmpC-producing *E. coli* with additional resistances to other antimicrobial classes. Despite their origin of different sampling districts, clones of two strains were removed from the analysis to reduce the bias of sampling the feces of the same dog. It is also possible, however, that the same clones occur in different animals. The appearance of identical clinical strains (IMT31286, IMT31333, and IMT31711) collected from different samples from different dogs at different time points underlines the success of certain clones in this clinical setting. Finding that same clone (IMT31356) outside the clinic supports the hypothesis of clinical-isolate transmission into the environment, possibly through one

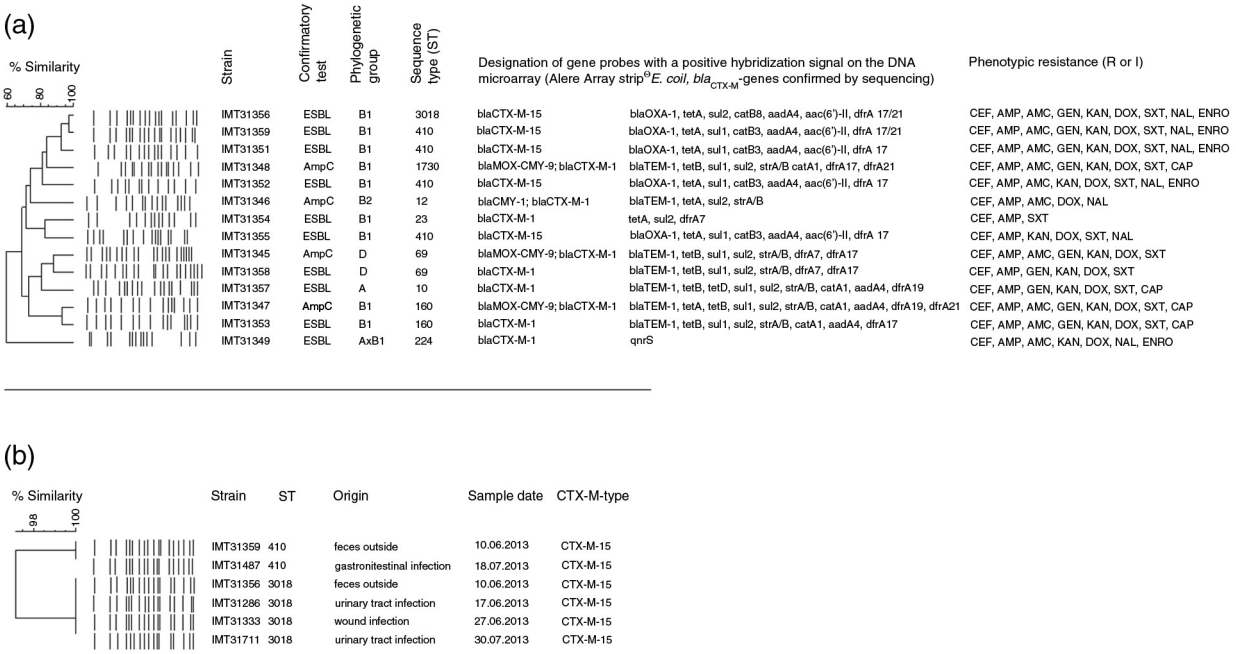


Fig. 1. (a) Phenotypic and genotypic typing results of ESBL- and AmpC-producing *E. coli* isolates based on a dendrogram (> *Xba*I-generated PFGE profiles after exclusion of double clones). Software: BioNumerics 6.6, Applied Maths, Belgium, cluster analysis of dice similarity indices based on the unweighted pair group method with arithmetic mean (UPGMA). (b) Comparison of clones from clinical dog isolates and extra-clinical fecal samples.
CEF: cefotaxime; AMP: ampicillin; AMC: amoxicillin–clavulanic acid; GEN: gentamicin; KAN: kanamycin; DOX: doxycycline; SXT: sulfamethoxazole–trimethoprim; CAP: chloramphenicol; NAL: nalidixic acid; ENRO: enrofloxacin; R: resistant; I: intermediate.

of the infected dogs or asymptomatically colonized animals. Interestingly, the corresponding clinical clones do not originate from gastrointestinal infections, but from urinary-tract and wound infections, demonstrating the clinical relevance of certain clones. Certain genetic resistance determinants found in the samples collected in this study, namely $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$, have recently been reported to be highly prevalent types of ESBL-colonizers in the healthy community in Bavaria (Germany) (18) and also in non-clinical and clinical samples from humans and companion animals in Europe (2, 4, 5, 19). Besides these gene types, detected STs including ST410 and ST224 are typically ESBL-associated STs, which have been frequently found in human medicine (5).

Although this pilot study faces epidemiological limitations due to a restricted sample size and non-identical sampling time procedures, the reservoir role of dog feces, which contain ESBL-producing *E. coli*, must not be underestimated.

The rate detected in this study reflects a slightly higher percentage of ESBL-/putative AmpC-producing *E. coli* than that of human isolates outside of hospital environments (3–10%) (18, 20, 21). The results might be influenced by the presence of the small-animal clinic in this area, which treats patients with antibiotics, thus selecting for resistance genes and therefore giving one explanation for high carriage of resistant bacteria in dogs taken for a walk on the campus. In addition, previous studies discussed a wash out of multiresistant bacteria from human and veterinary hospitals into the environment (11, 22, 23). However, dog owners visiting the clinic do not usually walk their dog as far as the adjoining areas where half the samples were collected, and other studies investigating healthy pets in Tunisia found comparable numbers of ESBL producers (3). Regardless, it is worth investigating further the extent to which dog feces in other public urban areas of Berlin, in the adjacent neighborhoods of veterinary clinics, and cities worldwide, contain multiresistant *E. coli*.

Conclusion

To best of the authors' knowledge, this is the first study estimating the rate of ESBL-/putative AmpC-producing *E. coli,* which have been isolated from environmental dog feces, and comparing these to isolates from a nearby small-animal clinic. As dogs often live close to humans and their zoonotic role as potential carriers of multiresistant strains should not be underestimated, their feces as a mechanism for transmission in environmental settings, particularly in close proximity to veterinary clinics, must be acknowledged and better understood. Dog owners in general but especially those of diseased dogs should be educated to remove the pet's feces immediately as other dogs' sniffing behavior might contribute to the zoonotic and environmental spread of ESBL-producing bacteria.

Despite the limits of this study's generalizability, it follows an integrated, 'One Health' approach that might serve as a model for future cross-cutting collaborations as well as opportunities for expanded epidemiological research and next-generation sequencing applications in the field of multiresistant bacteria.

Acknowledgements

We would like to thank the following for helping with the sampling and the analysis: K Oelgeschläger, A Schmidt, CA Bertram, B Gutjahr, J Kandzia, J Viernickel, O Wenske, S Wichert, S Winkler, S Woelke, L Niemann, and H Laube.

Conflict of interest and funding

The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

References

1. Pitout JD. Extraintestinal pathogenic *Escherichia coli*: a combination of virulence with antibiotic resistance. Front Microbiol 2012; 3: 9.
2. Hordijk J, Schoormans A, Kwakernaak M, Duim B, Broens E, Dierikx C, et al. High prevalence of fecal carriage of extended-spectrum beta-lactamase/AmpC-producing *Enterobacteriaceae* in cats and dogs. Front Microbiol 2013; 4: 274.
3. Sallem RB, Gharsa H, Slama KB, Rojo-Bezares B, Estepa V, Porres-Osante N, et al. First detection of CTX-M-1, CMY-2, and QnrB19 resistance mechanisms in fecal *Escherichia coli* isolates from healthy pets in Tunisia. Vector Borne Zoonotic Dis 2013; 13: 98–102.
4. Costa D, Poeta P, Brinas L, Saenz Y, Rodrigues J, Torres C. Detection of CTX-M-1 and TEM-52 beta-lactamases in *Escherichia coli* strains from healthy pets in Portugal. J Antimicrob Chemother 2004; 54: 960–1.
5. Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect 2012; 18: 646–55.
6. Procter TD, Pearl DL, Finley RL, Leonard EK, Janecko N, Reid-Smith RJ, et al. A cross-sectional study examining the prevalence and risk factors for anti-microbial-resistant generic *Escherichia coli* in domestic dogs that frequent dog parks in three cities in South-Western Ontario, Canada. Zoonoses Public Health 2014; 61: 250–9.
7. Berlin-Brandenburg Statistisches Landesamt (2013). Statistisches Jahrbuch. Berlin: Kulturbuchverlag GmbH.
8. Blouin DD. All in the family? Understanding the meaning of dogs and cats in the lives of American pet owners. PhD thesis, Indiana University, Bloomington, IN, 2008.
9. Walther B, Hermes J, Cuny C, Wieler LH, Vincze S, Abou Elnaga Y, et al. Sharing more than friendship – nasal colonization with coagulase-positive staphylococci (CPS) and co-habitation aspects of dogs and their owners. PLoS One 2012; 7: e35197.
10. Meyer E, Gastmeier P, Kola A, Schwab F. Pet animals and foreign travel are risk factors for colonisation with extended-spectrum beta-lactamase-producing *Escherichia coli*. Infection 2012; 40: 685–7.
11. Ghosh A, KuKanich K, Brown CE, Zurek L. Resident cats in small animal veterinary hospitals carry multi-drug resistant

enterococci and are likely involved cross-contamination of the hospital environment. Front Microbiol 2012; 3: 62.
12. Winkle S. Mikrobiologische und serologische Diagnostik. Jena: VEB Gustav Fischer Verlag; 1979.
13. CLSI (2008). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard. 3rd ed. Wayne, PA: CLSI.
14. Bogaerts P, Hujer AM, Naas T, de Castro RR, Endimiani A, Nordmann P, et al. Multicenter evaluation of a new DNA microarray for rapid detection of clinically relevant bla genes from beta-lactam-resistant gram-negative bacteria. Antimicrob Agents Chemother 2011; 55: 4457–60.
15. Ewers C, Grobbel M, Stamm I, Kopp PA, Diehl I, Semmler T, et al. Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum- beta -lactamase-producing *Escherichia coli* among companion animals. J Antimicrob Chemother 2010; 65: 651–60.
16. Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 2002; 40: 2153–62.
17. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol 2006; 60: 1136–51.
18. Valenza G, Nickel S, Pfeifer Y, Eller C, Krupa E, Lehner-Reindl V, et al. Extended-spectrum-beta-lactamase-producing *Escherichia coli* as intestinal colonizers in the German community. Antimicrob Agents Chemother 2014; 58: 1228–30.
19. Franiek N, Orth D, Grif K, Ewers C, Wieler LH, Thalhammer JG, et al. ESBL-producing *E. coli* and EHEC in dogs and cats in the Tyrol as possible source of human infection. Berl Munch Tierarztl Wochenschr 2012; 125: 469–75.
20. Valverde A, Coque TM, Sanchez-Moreno MP, Rollan A, Baquero F, Canton R. Dramatic increase in prevalence of fecal carriage of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* during nonoutbreak situations in Spain. J Clin Microbiol 2004; 42: 4769–75.
21. Stromdahl H, Tham J, Melander E, Walder M, Edquist PJ, Odenholt I. Prevalence of faecal ESBL carriage in the community and in a hospital setting in a county of Southern Sweden. Eur J Clin Microbiol Infect Dis 2011; 30: 1159–62.
22. Wellington EM, Boxall AB, Cross P, Feil EJ, Gaze WH, Hawkey PM, et al. The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. Lancet Infect Dis 2013; 13: 155–65.
23. Martinez JL. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. Proc Biol Sci 2009; 276: 2521–30.

4. DISCUSSION

With their ubiquity across various habitats and hosts and their role as indicator strains in fecal contamination of food and water, and their often multi-resistant phenotype, *E. coli* are a prime opportunity to study the global challenge of AMR. This not only applies for human and animal public health but also for settings with lower antimicrobial pressures, such as the environment and wildlife. Once washed out into these extra-clinical settings, clinically relevant resistant *E. coli* are part of hot spots in the means of genetic exchange of resistance factors, and possible infection sources and transmission origins for both humans and animals.

Within this thesis, ESBL-producing *E. coli* from different environmental origins were analyzed and compared to human and animal clinical samples, elucidating phylogenetic and clonal relationships and transmission scenarios of multi-resistant bacteria. This helps underline the importance of collaborations and communications in the fields of human, animal, and environmental health; the so-called One Health approach.

The discussion part starts with the detection rates of ESBL-producing *E. coli* in environmental dog feces and wild birds, the two settings addressed in the publications and in this thesis, followed by their further characterization with an emphasis on CTX-M-enzymes and multi-locus sequence types. Finally ESBL-producing ST410 clones from different settings and their zoonotic potential are discussed with respect to the One Health concept.

To fully characterize the diversity of a bacterial population, both the chromosomal background (ST, clone) and the episome (extrachromosomal content such as plasmids with resistance determinants) have to be analyzed, which results in a very complex overall evaluation.

4.1 Rates of ESBL-producing *E. coli* in environmental dog feces and wild birds

Studying ESBL-producing *E. coli* in rather unexplored habitats and hosts with subsequent clonal and phylogenetic analyses only makes sense once they are found to be prevalent there. Only detecting exceptions may not constitute a significant global public health threat. We have, indeed, found remarkable numbers of ESBL-producing *E. coli* in dog feces (10 % [I] [41]) and wild birds (7.5 %, including AmpC-producing *E. coli* [II] [42]). While not many studies address the occurrence of these bacteria in dog feces collected from the environment [148], ESBL-producing *E. coli* have been described in wild animals, especially birds. Their detection rate from different geographic regions is variable and ranges from 0.5 % in birds

from the Azorean Islands [11, 217] to 32 % in birds from the Iberian Peninsula [11, 39]. A recently published study found 59 % of birds positive for ESBL-carriage in Bangladesh [218]. The birds took their food from hospital waste, thus possibly showing these extremely high rates. In a study in which wild birds were sampled in Saxony-Anhalt in Germany, the approximate carriage rate of ESBL-producing *E. coli* was 5 % [12]. The slightly higher detection rate in our study (II) [42] may be a reflection of the more densely populated city of Berlin. The carriage rate in Saxony-Anhalt was similar to the one found from wild birds in scarcely populated areas in Mongolia [12], perhaps due to the migrating behavior of wild birds [11]. In contrast, Skurnik *et al.* [136] described a clearly anthropogenic related occurrence of resistant bacteria collected from animal feces meaning that the prevalence of these bugs increased with the animals exposure to humans. These diverse statements on the occurrence of antimicrobial resistant bacteria and their association to human activities underline the complex epidemiology of ESBL-producing *E. coli* across extra-clinical settings. In addition, data of the latter study date back a couple of years and the situation might be different meanwhile. Overall, in our study, highest numbers of ESBL-producing *E. coli* in wild birds were detected in mute swans (26.5 % [II]) underlining the literature's statement on waterfowl being one of the most important wild bird groups for ESBL-carriage [11], due to the confluence of several risk factors, including exposure to contaminated surface waters, and their potential to develop lifelong pick-up of resistant bacteria, which can often be up to twenty years. Berlin and Brandenburg are both known for their numerous lakes and rivers, creating a melting pot for the interference of humans and wild animals, especially waterfowl.

The carriage rate in wild birds [II] is comparable to the presence of ESBL-producing *E. coli* in the healthy German community, where intestinal colonization rates were estimated to be 6.3 % [24]. Rates in hospitalized human patients and their household contacts are usually higher (approximately 16 %) [10], which provides a first explanation for why the detection rates found in the dog feces study [I] [41] were higher than in wild birds: i) dogs are generally in much closer contact with humans than wild birds and ii) the dog feces in this study were collected in close proximity to a veterinary clinic. ESBL-producing isolates from the first study [I], however, were found in feces collected from adjoining residential neighborhoods of the veterinary campus, possibly from dogs, which did not visit the small animal clinic and which were asymptomatic carriers as dog owners of hospitalized patients would probably not walk their dogs as far as these areas [41]. The detection rate of ESBL-producing *E. coli* in clinical samples from dogs and cats in a study from France was shown to be 3.7 % [28], a lot lower than the one found in our study, strengthening the case for revisiting supposedly

underestimated fecal samples from the environment. Only few studies have determined the occurrence of ESBL-producing *E. coli* in healthy dogs [31]. The rate found in our study (I) [41] somehow fits in the colonization rates described, ranging from 0 % from a 2009 study in Canada [151] over 5.8 % from a 2011 study in South Korea [219] and 12.2 % in Tunisia [33] up to 22 % in Kenya [220].

In general, comparing bacterial rates from different countries and backgrounds presents difficulties due to differing epidemiological approaches, sample schemes, laboratory procedures etcetera. A putative solution calls for longitudinal surveillance strategies with clearly defined habitats and harmonized sampling, detection and evaluation methods.

In summary, the detected high rates in both environmental dog feces (I) and wild birds (II) were too high to be simply a random finding of ESBL-producing *E. coli* in these settings, deserving further investigations.

4.2 ESBLs of ESBL-producing *E. coli* in environmental dog feces and wild birds

The CTX-types of ESBL-producing *E. coli* from the two groups were determined to be one of the first characterization steps to assess basic similarities. CTX-M-15 and CTX-M-1 were the only detected ESBL-types both in environmental dog feces (I) [41] and wild birds (II) [42], reinforcing the worldwide success of these ESBL-types in different environments [32]. Looking into their dissemination among different hosts, the carriage of CTX-M-15-producing bacteria seems to be especially associated with humans [221] and companion animals [150], while only few studies report their detection in livestock [221-223]. On the contrary, CTX-M-1 is less frequently found in humans, yet, in recent years numbers of this type are on the rise [24]. It is by far the most frequently detected enzyme in various livestock animals in Europe [221]. Sixty percent of *E. coli* from wild birds in our study (II) [42] were positive for this ESBL-type, perhaps explained by their avian hosts often proximate living to farms and fields. The other 40 %, however, were assigned to the $bla_{\text{CTX-M-15}}$ gene, hence the statement on a clear correlation would go too far. Given the results in environmental dog feces (I) [41], the detected ESBLs are the ones apparent in companion animals [32], which was expected. The association of especially CTX-M-15 with human types is explainable: by the often close contact between owners and their pet [164]. Interestingly, in environmental and wildlife isolates, the variety of ESBL-enzymes detected is generally lower than the one in human or veterinary medicine. This could be due to limited study numbers regarding ESBL-producing

bacteria in wildlife but also because of the success of certain ESBL-types (e.g. CTX-M-15 or CTX-M-1) in the environment.

A report from Sweden states that ESBL-genes (including *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-1}}$) from *E. coli* in the Swedish community and sewage water were approximately 90 % similar to those collected from the environment in contrary to those from farm animals and food products [224]. This contradicts the assumption that food-producing animals are the only main origin for environmental pollution with multi-resistant bacteria. In addition, our data suggest a far more complex situation of the distribution of ESBL-producing *E. coli* in different hosts and habitats. To mainly "blame" animal husbandry and related professional categories should perhaps be reconsidered.

In general, the often shared ESBL-types, be it from humans or livestock/domestic animals, compared to the environment and wildlife might provide a clue in better understanding the ongoing transmission of multi-resistant bacteria between these different settings. The application of CTX-M-data to make a strong statement on origin and relatedness of multi-resistant bacteria, however, is questionable. Most ESBL-types occur in all kinds of different settings anyway, either from humans, domestic animals or livestock.

4.3 STs of ESBL-producing *E. coli* in environmental dog feces and wild birds

To spot further similarities among ESBL-producing *E. coli*, we then multi-locus sequence typed isolates. We found a variety of different STs in environmental dog feces (I) [41] and wild birds (II) [42] including ST10 (1 x in dog feces), ST131 (1 x in wild bird), ST167 (1 x in wild bird), ST224 (1 x in dog feces and 1 x in wild bird), ST405 (2 x in wild birds), ST410 (4 x in dog feces and 3 x in wild birds) and ST617 (3 x in wild birds). Interestingly, these listed STs are exactly the ones often described in human and veterinary medicine [32], which was expected for the dog feces isolates (I) but is rather surprising and an interesting fact for the avian isolates (II) implying that our collected isolates might be clinically relevant rather than representing arbitrary sequence types which had acquired ESBL-plasmids randomly. In addition, many of these STs are described to often produce ESBL-enzymes [11], for example ST10, which is associated with their production in humans and companion animals worldwide [32]. Besides ST10, especially ST131 attracts attention all over the world, known for its spread not only in humans [118] and companion animals [225] but also wild birds [138] and the environment, such as wastewater [35].

We focused our continuative detailed analyses on ST410, a sequence type occurring in both the environmental fecal samples (I), one compared canine clinical sample (1 x [I]) [41], wild birds (II) as well as in those of human clinical origin (3 x [II]) from our second study [42]. Our suggestion that this sequence type might emerge globally in various habitats and hosts as new pandemic, successful clone is underlined by its detection in several settings worldwide, for example in humans [226], poultry [227], companion animals [228] and rivers [229]. Interestingly, ST410 belongs to phylogenetic group B1 known for harboring less virulent isolates, in the contrary to phylogenetic group B2, for instance [191, 192, 230]. This fact pleads for other important factors beyond virulence that contribute to its global spread. In general, the global success of pandemic sequence types like ST131 from group B2 or ST648 from group D is discussed to be a combination of phylogenetic background, virulence and antimicrobial resistance [77], which therefore might not completely apply for ST410.

The finding of human- and animal-associated STs in our environmental (I) and wildlife (II) samples fosters the hypothesis of an ongoing interspecies transmission between different hosts and habitats. In fact, for ST410, it was not only the shared sequence type but a clone, as discussed in the next chapter. Together with the similar ESBL-types this is just another important piece of the puzzle to elucidate what actually happens regarding the dissemination of multi-resistant bacteria in different environments. Here, MLST is a good tool to narrow the pool of interesting isolates down to those subsequently chosen for a deeper analysis. A clonal investigation, however, was not possible. Techniques like macrorestriction analysis constituted the next required step.

4.4 Clones of ESBL-producing *E. coli* of ST410

The base of the next stage of elucidating clonal and phylogenetic similarities and transmission scenarios of multi-resistant bacteria was to analyze representatives of these bugs in depth: ESBL-producing *E. coli* of ST410. This ST was one of the sequence types found in our two sample groups (environmental dog feces [I] [41] and wild birds [II] [42]) and it also occurred in one canine clinical sample from the first study (I) and in three samples from a human clinical context (second study [II]). In general, one cannot assume that all isolates from the same ST represent the same clone, which made it particularly surprising that the overall ten ESBL-producing *E. coli* isolates of ST410 showed almost identical macrorestriction patterns after performing pulsed-field gel electrophoresis. *E. coli* strains are commonly assigned to one clonal PFGE group, when their patterns show 85 % of similarity [231]. Given the overall

similarity of at least 94 % in our ten ST410 isolates, this already indicates their very close relatedness. PFGE is advantageous in the study of bacterial outbreaks, but has its disadvantages. For example the possibility of only detecting SNPs when they are exactly located at the enzyme-restriction site may obfuscate its interpretation. It may also miss mobile genetic elements and SNPs up to several hundred base pairs. Until recently, it was regarded as the "gold standard" to perform clonal analyses and was used to find clonally related isolates within ST131 and ST648 from different settings [29, 150]. Not wanting to rely solely on this method, we employed NGS applications to analyze WGS data. This allowed a by far higher resolution and helped in making a more precise statement on the relatedness of our isolates, down to the level of even detecting variations in single base pairs.

The analysis of the whole genomes resulted in the maximum common genome, the gene clusters present in all ten isolates, which was used to identify single-nucleotide polymorphism sites, the base to generate a phylogenetic tree (II). Strikingly, only few numbers of SNPs were found between the different isolates, always less than 100 within a clade and not more than 1767 between the clades (Cluster I and Cluster II [II]) [42]. So far, these data clearly provided the strongest evidence for the clonal relatedness of the ten ESBL-producing *E. coli* of ST410 from different habitats and hosts. Whether their clonality is due to a microevolution in the restricted sample location of Berlin and Brandenburg or a successful emergence and transmission of a certain clone of ST410, needs to be further investigated. The sampling period of our isolates ranging from 2009 to 2014 might rather support their success and recent, maybe still ongoing transmission events between different habitats and hosts. Our macrorestriction data demonstrate a considerable reflection of the NGS results, thus proving their applicable reliability.

The original title of the first publication (I) "Putative connection between zoonotic multiresistant extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in dog feces from a veterinary campus and clinical isolates from dogs" [41] should be reconsidered in retrospect. After calling the single base variations of the four ST410 isolates from this study, we found zero SNPs between IMT13159 (environmental dog feces) and IMT31487 (canine clinical isolate) and four SNPs between IMT31351 and IMT31487 clearly highlighting the clonal character of these isolate combinations. Although, in this study, we principally discussed sequence type 3018 as subject of the association, ST410 was included as well and looking at the putative connection in this overall new context, the term "putative" could be removed. A connection of isolates originating from environmental dog feces with those collected from a small animal clinic in the same area of Berlin was proven.

Taking together these results and the additional similar large plasmids, similar resistance phenotypes and similar serotypes reinforced the clonal character of the ten isolates of ST410, importantly contributing to a more complete picture of this putative new clone and also demonstrating how different *E. coli* characterization methods should be mutually integrated.

4.5 Zoonotic potential of ESBL-producing *E. coli* in a One Health perspective

The fight against antimicrobial resistance was put on the agenda of the G7 summit in June of 2015, highlighting the urgency and importance of global collaboration and action (http://www.leopoldina.org/uploads/tx_leopublication/2015_G7_Statement_Infectious_Diseases.pdf). While AMR will not be completely eradicated, multidisciplinary efforts can limit risks for public health as pointed out in the "UK Five Year Antimicrobial Resistance Strategy 2013 to 2018" [232]. International cooperation is only one dimension of this approach; all health sectors have to be included proportionately, including environmental health the same as human and veterinary public health. The collaborations and communications in these fields are summarized as the One Health approach, where zoonotic infectious diseases constitute a main focus.

This thesis aimed to contribute to the characterization of zoonotic and AMR ESBL-producing *E. coli* in rarely investigated settings such as wildlife (II) and environmental dog feces (I) and to the understanding of the occurrence of clones of these bugs in different habitats (II). As pointed out in the two studies and preceding discussion, highly genetically related ESBL-producing *E. coli* of sequence type 410, differing only by few SNPs, were detected in environmental dog feces, a canine clinical sample, wild birds and human clinical isolates thus making an appropriate small set to study phylogenetic and clonal relationships of these bacteria in a One Health background and providing the basis for future large-scale investigations. All of the ten isolates were not only resistant against beta-lactams but at least three classes of antimicrobials, thereby having a multi-resistant phenotype. The origins of isolates, namely wild birds (II) and dog feces collected from the environment (I), represent a perfect non-point infection source for other animals and humans, be it through the direct contact with bird and dog droppings or indirect detours such as water, farmland and farm products and soil. As another example, resistance determinants and AMR *E. coli* are also found in children's playgrounds and sand pits [233, 234], which is not surprising and which is likely explained by their often proximity to public parks, where dogs are taken for walks and

wild birds populate. These settings display an especially alarming environment as young individuals with weak immune systems are exposed to AMR and infectious bugs.

One key area in the above mentioned strategy, which needs to be achieved to fight AMR, is surveillance, which "quickly identifies new threats or changing patterns in resistance" [232]. Our results contribute to the overall understanding of these patterns with an emphasis on ESBL-producing *E. coli* as an emerging threat. Other infectious agents are important as well, with *Klebsiella pneumoniae, Staphylococcus aureus* and *Streptococcus pneumoniae* just a few to mention [46]. Instead of only having to fight against the serious problem of multi-resistance, factors leading to AMR have to be identified and infections prevented, which includes better hygiene, access to clean water and sanitation, infection control and vaccination [46]. Clearly, the ubiquitously present threat of AMR determinants and agents in environmental settings seems underestimated, thus calling not only for improved prescription and treatment awareness among human and animal health sectors and development of new therapeutic options, but also for the responsibility of every single private person; not every infection needs to be treated with antimicrobials and AMR infection sources, such as dog feces, should be removed by the owner [41].

Although we found clinically relevant clones of ESBL-producing *E. coli* of ST410 in different habitats and hosts, permitting interpretation on recent, maybe ongoing transmission scenarios, the clear determination of the original, first source remains difficult for a range of methodological reasons, including limited sample sizes. Our studies are pilot studies with results strongly highlighting the importance of future research in environmental microbiology, in-depth epidemiology and in elucidating mechanisms of successful clonal lineages of certain *E. coli* STs.

One Health is not just a term, it has to be implemented. Efforts should be taken to combat the challenge of antimicrobial resistant bacteria collaboratively, rather than accusing each other, be it human medical doctors, veterinarians, politicians or environmentalists.

5. CONCLUSION AND OUTLOOK

Several conclusions can be drawn by interpreting our results:

- The application of an appropriate set of ESBL-producing *E. coli* isolates from different habitats and hosts enables the study of detection rates, phylogenetic and clonal relationships as well as transmissions between the different (extra-clinical) settings. Given that this work only addressed a small random sample set, one must wonder what large-scale epidemiological studies might reveal concerning transmission scenarios. Our findings are likely not incidental, however, this has to be proven in the future.

- The finding of a potentially new clone of ST410 *E. coli* putatively circulating between different habitats and hosts raises the question of why certain clones are successful worldwide, in habitats of both high and low antimicrobial impacts. This might be due to reasons beyond antimicrobial resistance, such as metabolic/virulence-associated factors in combination with the phylogenetic background of these isolates. Functional and in-depth molecular (sequencing) analyses will help detect underlying mechanisms to elucidate contributing factors.

- The findings underpin the utmost importance of collaboration and action across the fields of human, animal and environmental health; embodied by the One Health concept. Decades of mostly solitary research and development approaches in these sectors should be left behind to fight the global challenge of antimicrobial resistance jointly. This should specifically include large-scale surveillance and control functions, such as the holistic "Integrated Surveillance", which "envisages all surveillance activities in a country as a common public service that carries out many functions using similar structures, processes and personnel" [235].

- The still open question: who is egg and who is chicken and which comes first? Is the origin of transmission of clinically relevant clones the clinical or the environmental setting? This has to be addressed in future long-scale and comprehensive epidemiological studies.

6. SUMMARY

Clinically relevant extended-spectrum beta-lactamase (ESBL)-producing multi-resistant *Escherichia (E.) coli* are increasingly detected in various habitats, not only in a human and veterinary clinical context but also in extra-clinical settings such as communities, the environment and wildlife. Infections including diarrhea, septicemia, pneumonia, and wound and urinary tract infections these bugs cause are not only severe but also difficult to treat due to limitations in antimicrobial therapy possibilities.

This thesis is based on a total of 100 samples from environmental dog feces and 320 cloacal samples from wild birds, which were collected in Berlin, Germany, between 2008 and 2014. Of these 10 % (dogs) respectively 7.5 % (birds) harbored ESBL-producing *E. coli*. In addition, eleven canine clinical and 40 human clinical (bacteremia) ESBL-producing *E. coli* isolates from the same area were included and pheno- and genotypically analyzed. To determine the phylogenetic population structure of ESBL-producing isolates, multi-locus sequence typing (MLST) was performed. The sequence type (ST) occurring in all sample groups was ST410. All ST410 isolates were initially clonally analyzed using pulsed-field gel electrophoresis (PFGE). Ten isolates from one clonal PFGE group with identical or almost identical macrorestriction patterns were then chosen for whole-genome sequencing using the MiSeq platform (Illumina). Following raw data reprocessing through standard bioinformatics pipelines it was possible to perform detailed phylogenetic and clonal as well as molecular analyses, again including both chromosomal and episomal (plasmids) determinants.

Besides ST410, various sequence types frequently described in both humans and animals worldwide were detected in environmental dog feces and wild birds (ST10, ST131, ST224) with all isolates harboring the typical beta-lactamase (*bla*)-gene $bla_{\text{CTX-M-1}}$ or $bla_{\text{CTX-M-15}}$. Within the ten ST410 strains from different hosts in the same region, almost genetically identical isolates were identified. As some of the isolates differed by a few single-nucleotide polymorphisms (SNPs) only, the study gives initial evidence for an ongoing interspecies transmission of a putative successful new *E. coli* clone of ST410 between avian wildlife, humans, companion animals and the environment. This underlines the zoonotic potential of ESBL-producing *E. coli* as well as the mandatory nature of the One Health approach to address the threat of antimicrobial resistance. Future investigations call for long-scale and comprehensive epidemiological studies and functional analyses to reinforce the proposed transmission scenarios and to identify infection sources and underlying molecular mechanisms of successful pandemic clones.

7. ZUSAMMENFASSUNG

Die molekulare Analyse von ESBL-bildenden *Escherichia coli* aus verschiedenen Habitaten gewährt Einblicke in Phylogenie, klonale Beziehungen und Transmissionsszenarien

Klinisch relevante extended-Spektrum beta-Laktamase-bildende multi-resistente *Escherichia (E.) coli* werden zunehmend in verschiedenen Milieus detektiert, nicht nur in einem human- und veterinärmedizinischen Kontext, sondern auch in extra-klinischen Umfeldern wie in der Gesellschaft, in Wildtieren und in der Umwelt. Infektionen, einschließlich Diarrhoe, Septikämie, Pneumonie sowie Wund- und Harnwegsinfektionen, welche diese Bakterien verursachen, sind nicht nur schwerwiegend, sondern durch die Einschränkungen bei antimikrobiellen Therapiemöglichkeiten auch schwierig zu behandeln.

Diese Doktorarbeit basiert auf insgesamt 100 Proben aus Umwelthundekot und 320 Kloakenproben aus Wildvögeln in Berlin, Deutschland, die zwischen 2008 und 2014 gesammelt wurden. Von diesen trugen 10 % (Hunde) beziehungsweise 7.5 % (Wildvögel) ESBL-bildende *E. coli*. Zusätzlich wurden elf Hunde-klinische und 40 humane klinische (Bakteriämie) ESBL-bildende *E. coli* Isolate aus derselben Region berücksichtigt und pheno- und genotypisch charakterisiert. Um die phylogenetische Populationsstruktur der ESBL-bildenden Isolate zu bestimmen, wurde eine Multilokus Sequenztypisierung (MLST) durchgeführt. Der in allen Probengruppen vorkommende Sequenztyp (ST) war ST410. Alle ST410 Isolate wurden zunächst mittels Pulsfeld Gelelektrophorese (PFGE) klonal analysiert. Zehn Isolate aus einer klonalen PFGE Gruppe mit identischen oder fast identischen Makrorestriktionsmustern wurden dann für eine Gesamtgenomsequenzierung mittels MiSeq Plattform (Illumina) ausgewählt. Folgend einer Neubearbeitung von Rohdaten durch standardisierte bioinformatische Pipelines war es möglich, detaillierte phylogenetische und klonale sowie molekulare Analysen durchzuführen, wieder unter Einschluss von sowohl chromosomalen als auch episomalen (Plasmide) Determinanten.

Neben ST410 wurden verschiedene Sequenztypen, die häufig sowohl in Menschen als auch Tieren weltweit beschrieben sind, in Umwelthundekot und Wildvögeln detektiert (ST10, ST131, ST224). Alle Isolate enthielten das typische beta-Laktamase (*bla*)-Gen $bla_{\text{CTX-M-1}}$ oder $bla_{\text{CTX-M-15}}$. Innerhalb der zehn ST410 Stämme aus verschiedenen Wirten in der gleichen Region wurden fast genetisch identische Isolate identifiziert. Da sich manche der Isolate nur in einigen Einzelnukleotid Polymorphismen (SNP) unterschieden, gibt diese Studie einen ersten Hinweis auf eine permanente Inter-Spezies Transmission von mutmaßlich

erfolgreichen neuen *E. coli* Klonen von ST410 zwischen aviären Wildtieren, Menschen, Haustieren und der Umwelt. Dies unterstreicht das zoonotische Potential von ESBL-produzierenden *E. coli* sowie die dringende Notwendigkeit des One Health Ansatzes, um die Gefahr der antimikrobiellen Resistenz anzugehen. Zukünftige Untersuchungen erfordern groß angelegte und umfassende epidemiologische Studien und funktionelle Analysen, um die hier unterbreitete Aussage über Transmissionsszenarien zu bekräftigen und um zugrundeliegende molekulare Mechanismen von erfolgreichen pandemischen Klonen zu identifizieren.

8. REFERENCES

1. **Atlas R. and Maloy S.**: *One Health: People, Animals, and the Environment* 2014, Washington DC (USA): ASM Press.
2. **Institute of Medicine**: *Improving Food Safety Through a One Health Approach: Workshop Summary*. 2012, Washington DC (USA): National Academies Press.
3. **Franiczek R. *et al.***: ESBL-producing *Escherichia coli* isolated from children with acute diarrhea - antimicrobial susceptibility, adherence patterns and phylogenetic background. Adv Clin Exp Med, 2012. 21(2): p. 187-192.
4. **Kim Y.K. *et al.***: Bloodstream infections by extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in children: Epidemiology and clinical outcome. Antimicrob Agents Chemother, 2002. 46(5): p. 1481-1491.
5. **Calbo E. *et al.***: Risk factors for community-onset urinary tract infections due to *Escherichia coli* harbouring extended-spectrum beta-lactamases. J Antimicrob Chemother, 2006. 57(4): p. 780-783.
6. **Teshager T. *et al.***: Isolation of an SHV-12 beta -lactamase-producing *Escherichia coli* strain from a dog with recurrent urinary tract infections. Antimicrob Agents Chemother, 2000. 44(12): p. 3483-3484.
7. **Nathisuwan S. *et al.***: Extended-spectrum beta-lactamases: epidemiology, detection, and treatment. Pharmacotherapy, 2001. 21(8): p. 920-928.
8. **Doi Y. *et al.***: Community-associated extended-spectrum beta-lactamase-producing *Escherichia coli* infection in the United States. Clin Infect Dis, 2013. 56(5): p. 641-648.
9. **Woodford N. *et al.***: Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum beta-lactamases in the UK. J Antimicrob Chemother, 2004. 54(4): p. 735-743.
10. **Valverde A. *et al.***: High rate of intestinal colonization with extended-spectrum-beta-lactamase-producing organisms in household contacts of infected community patients. J Clin Microbiol, 2008. 46(8): p. 2796-2799.
11. **Guenther S.**: *Multi-resistant Escherichia coli from wildlife* 2015, Berlin (Germany): Habilitation thesis Freie Universität Berlin.
12. **Guenther S. *et al.***: Comparable high rates of extended-spectrum-beta-lactamase-producing *Escherichia coli* in birds of prey from Germany and Mongolia. PLoS One, 2012. 7(12): p. e53039.
13. **Literak I. *et al.***: Reservoirs of antibiotic-resistant Enterobacteriaceae among animals sympatric to humans in Senegal: extended-spectrum beta-lactamases in bacteria in a black rat (*Rattus rattus*). Afr J Microbiol Res, 2009. 3(11): p. 751-754.
14. **Guenther S. *et al.***: Extended-spectrum beta-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution? Front Microbiol, 2011. 2: p. 246.
15. **Dolejska M. *et al.***: Antibiotic-resistant *Salmonella* and *Escherichia coli* isolates with integrons and extended-spectrum beta-lactamases in surface water and sympatric black-headed gulls. J Appl Microbiol, 2009. 106(6): p. 1941-1950.
16. **Wellington E.M.H. *et al.***: The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. Lancet Infect Dis, 2013. 13(2): p. 155-165.
17. **Berendonk T.U. *et al.***: Tackling antibiotic resistance: the environmental framework. Nat Rev Micro, 2015. 13(5): p. 310-317.
18. **Gniadkowski M. *et al.***: Cefotaxime-resistant Enterobacteriaceae isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing beta-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. Antimicrob Agents Chemother, 1998. 42(4): p. 827-832.
19. **Smet A. *et al.***: Characterization of extended-spectrum beta-lactamases produced by *Escherichia coli* isolated from hospitalized and nonhospitalized patients: emergence of CTX-M-15-producing strains causing urinary tract infections. Microb Drug Resist, 2010. 16(2): p. 129-134.

20. **Van Der Bij A.K. *et al.***: Clinical and molecular characteristics of extended-spectrum-beta-lactamase-producing *Escherichia coli* causing bacteremia in the Rotterdam Area, Netherlands. Antimicrob Agents Chemother, 2011. 55(7): p. 3576-3578.
21. **Mshana S.E. *et al.***: Conjugative IncFI plasmids carrying CTX-M-15 among *Escherichia coli* ESBL producing isolates at a University hospital in Germany. BMC Infect Dis, 2009. 9: p. 97.
22. **Ben Slama K. *et al.***: Diversity of genetic lineages among CTX-M-15 and CTX-M-14 producing *Escherichia coli* strains in a Tunisian hospital. Curr Microbiol, 2011. 62(6): p. 1794-1801.
23. **Johnson J.R. *et al.***: *Escherichia coli* sequence type ST131 as the major cause of serious multidrug-resistant *E. coli* infections in the United States. Clin Infect Dis, 2010. 51(3): p. 286-294.
24. **Valenza G. *et al.***: Extended-spectrum-beta-lactamase-producing *Escherichia coli* as intestinal colonizers in the German community. Antimicrob Agents Chemother, 2014. 58(2): p. 1228-1230.
25. **Mshana S.E. *et al.***: Multiple ST clonal complexes, with a predominance of ST131, of *Escherichia coli* harbouring blaCTX-M-15 in a tertiary hospital in Tanzania. Clin Microbiol Infect, 2011. 17(8): p. 1279-1282.
26. **Carattoli A.**: Animal reservoirs for extended spectrum beta-lactamase producers. Clin Microbiol Infect, 2008. 1: p. 117-123.
27. **Vo A.T. *et al.***: Characteristics of extended-spectrum cephalosporin-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates from horses. Vet Microbiol, 2007. 124(3-4): p. 248-255.
28. **Dahmen S. *et al.***: Characterization of bla(CTX-M) IncFII plasmids and clones of *Escherichia coli* from pets in France. J Antimicrob Chemother, 2013. 68(12): p. 2797-2801.
29. **Ewers C. *et al.***: CTX-M-15-D-ST648 *Escherichia coli* from companion animals and horses: another pandemic clone combining multiresistance and extraintestinal virulence? J Antimicrob Chemother, 2014. 69(5): p. 1224-1230.
30. **Huber H. *et al.***: ESBL-producing uropathogenic *Escherichia coli* isolated from dogs and cats in Switzerland. Vet Microbiol, 2013. 162(2-4): p. 992-996.
31. **Rubin J.E. and Pitout J.D.D.**: Extended-spectrum beta-lactamase, carbapenemase and AmpC producing Enterobacteriaceae in companion animals. Vet Microbiol, 2014. 170(1-2): p. 10-18.
32. **Ewers C. *et al.***: Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect, 2012. 18(7): p. 646-655.
33. **Sallem R.B. *et al.***: First detection of CTX-M-1, CMY-2, and QnrB19 resistance mechanisms in fecal *Escherichia coli* isolates from healthy pets in Tunisia. Vector Borne Zoonotic Dis, 2013. 13(2): p. 98-102.
34. **Hordijk J. *et al.***: High prevalence of fecal carriage of extended-spectrum beta-lactamase/AmpC-producing Enterobacteriaceae in cats and dogs. Front Microbiol, 2013. 4: p. 242.
35. **Dolejska M. *et al.***: CTX-M-15-producing *Escherichia coli* clone B2-O25b-ST131 and *Klebsiella spp.* isolates in municipal wastewater treatment plant effluents. J Antimicrob Chemother, 2011. 66(12): p. 2784-2790.
36. **Haque A. *et al.***: ESBL-producing Enterobacteriaceae in environmental water in Dhaka, Bangladesh. J Infect Chemother, 2014. 20(11): p. 735-737.
37. **Literak I. *et al.***: Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes encoding the extended-spectrum beta-lactamase and QnrS, in waterbirds on the Baltic Sea Coast of Poland. Appl Environ Microbiol, 2010. 76(24): p. 8126-8134.
38. **Guenther S. *et al.***: CTX-M-15-type extended-spectrum beta-lactamases-producing *Escherichia coli* from wild birds in Germany. Env Microbiol Rep, 2010. 2(5): p. 641-645.
39. **Simoes R.R. *et al.***: Seagulls and beaches as reservoirs for multidrug-resistant *Escherichia coli*. Emerg Infect Dis, 2010. 16(1): p. 110-112.
40. **Guenther S. *et al.***: First insights into antimicrobial resistance among faecal *Escherichia coli* isolates from small wild mammals in rural areas. Sci Total Environ, 2010. 408(17): p. 3519-3522.

41. **Schaufler K. *et al.***: Putative connection between zoonotic multiresistant extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in dog feces from a veterinary campus and clinical isolates from dogs. Infect Ecol Epidemiol, 2015. 5: p. 25334.
42. **Schaufler K. *et al.***: Clonal spread and interspecies transmission of clinically relevant ESBL-producing *Escherichia coli* of ST410 – another successful pandemic clone? FEMS Microbiol Ecol, 2015.
43. **CLSI**: *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard - Third Edition (Document M31-A3)*. 2008, Wayne (USA).
44. **Nationale Akademie der Leopoldina**: *Antibiotika-Forschung: Probleme und Perspektiven*. 2013, Berlin (Germany): De Gruyter.
45. **White A.R. and BSAC Working Party Urgent Need**: Effective antibacterials: at what cost? The economics of antibacterial resistance and its control. J Antimicrob Chemother, 2011. 66(9): p. 1948-1953.
46. **WHO**: *Antimicrobial Resistance: Global Report on Surveillance* 2014, New York (USA).
47. **Schwarz S. *et al.***: Assessing the antimicrobial susceptibility of bacteria obtained from animals. Vet Microbiol, 2010. 141(1-2): p. 1-4.
48. **Levy S.B.**: Factors impacting on the problem of antibiotic resistance. J Antimicrob Chemother, 2002. 49(1): p. 25-30.
49. **D'costa V.M. *et al.***: Antibiotic resistance is ancient. Nature, 2011. 477(7365): p. 457-461.
50. **Davies J. and Davies D.**: Origins and evolution of antibiotic resistance. Microbiol Mol Biol R, 2010. 74(3): p. 417-433.
51. **Abraham E.P. and Chain E.**: An enzyme from bacteria able to destroy penicillin. 1940. Rev Infect Dis, 1988. 10(4): p. 677-678.
52. **Forsberg K.J. *et al.***: The shared antibiotic resistome of soil bacteria and human pathogens. Science, 2012. 337(6098): p. 1107-1111.
53. **Finley R.L. *et al.***: The scourge of antibiotic resistance: the important role of the environment. Clin Infect Dis, 2013. 57(5): p. 704-10.
54. **Rolle M. and Mayr A.**: *Medizinische Mikrobiologie, Infektions- und Seuchenlehre*. 2007, Stuttgart (Germany): Enke-Verlag.
55. **SVARM**: *Swedish Veterinary Antimicrobial Resistance Monitoring*. 2011, Uppsala (Sweden): The National Veterinary Institute, SVA.
56. **DANMAP**: *Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Food Animals, Foods and Humans in Denmark*. 2007, Copenhagen (Denmark).
57. **BVL**: *Dritte Datenerhebung zur Antibiotikaabgabe in der Tiermedizin* 2014, Berlin (Germany).
58. **Kresken M.**: GERMAP 2008-Resistance monitoring in Germany: antibiotic use and occurrence of resistance in human medicine. J Vet Pharmacol Ther, 2009. 32: p. 51-51.
59. **Gräfe U.**: *Biochemie der Antibiotika* 1992, Heidelberg (Germany): Spektrum Akademischer Verlag
60. **Fleming A.**: On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae*. B World Health Organ, 2001. 79(8): p. 780-790.
61. **Papp-Wallace K.M. *et al.***: Carbapenems: Past, Present, and Future. Antimicrob Agents Chemother, 2011. 55(11): p. 4943-4960.
62. **Bradford P.A.**: Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev, 2001. 14(4): p. 933-951.
63. **Abraham S. *et al.***: Carbapenemase-producing bacteria in companion animals: a public health concern on the horizon. J Antimicrob Chemother, 2014. 69(5): p. 1155-1157.
64. **Frei H.-H. and Löscher W.**: *Lehrbuch der Pharmakologie und Toxikologie für die Veterinärmedizin*. 2002, Stuttgart (Germany): Enke Verlag.
65. **Simon C. and W. S.**: *Antibiotikatherapie in Klinik und Praxis*. 1993, Stuttgart (Germany): Schattauer.

66. **Livermore D.M.**: Beta-Lactamases in laboratory and clinical resistance. Clin Microbiol Rev, 1995. 8(4): p. 557-584.
67. **Hedge P.J. and Spratt B.G.**: Resistance to beta-lactam antibiotics by re-modelling the active-site of an *Escherichia coli* penicillin-binding protein. Nature, 1985. 318(6045): p. 478-480.
68. **Zapun A. *et al.***: Penicillin-binding proteins and beta-lactam resistance. Fems Microbiol Rev, 2008. 32(2): p. 361-385.
69. **Paulsen I.T. *et al.***: Proton-dependent multidrug efflux systems. Microbiol Rev, 1996. 60(4): p. 575-608.
70. **Gotoh N. *et al.***: The outer-membrane protein Oprm of *Pseudomonas-Aeruginosa* is encoded by Oprk of the Mexa-Mexb-Oprk multidrug-resistance operon. Antimicrob Agents Chemother, 1995. 39(11): p. 2567-2569.
71. **Beceiro A. *et al.***: Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? Clin Microbiol Rev, 2013. 26(2): p. 185-230.
72. **Ambler R.P.**: The structure of beta-lactamases. Philos Trans R Soc Lond B Biol Sci, 1980. 289(1036): p. 321-331.
73. **Bush K. *et al.***: A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother, 1995. 39(6): p. 1211-1233.
74. **Gniadkowski M.**: Evolution and epidemiology of extended-spectrum beta-lactamases (ESBLs) and ESBL-producing microorganisms. Clin Microbiol Infect, 2001. 7(11): p. 597-608.
75. **Knothe H. *et al.***: Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection, 1983. 11(6): p. 315-317.
76. **Sturenburg E. and Mack D.**: Extended-spectrum beta-lactamases: implications for the clinical microbiology laboratory, therapy, and infection control. J Infect, 2003. 47(4): p. 273-295.
77. **Pitout J.D.**: Extraintestinal pathogenic *Escherichia coli*: a combination of virulence with antibiotic resistance. Front Microbiol, 2012. 3: p. 9.
78. **Pitout J.D. and Laupland K.B.**: Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis, 2008. 8(3): p. 159-266.
79. **Bonnet R.**: Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother, 2004. 48(1): p. 1-14.
80. **Schwaber M.J. *et al.***: High levels of antimicrobial coresistance among extended-spectrum-beta-lactamase-producing Enterobacteriaceae. Antimicrob Agents Chemother, 2005. 49(5): p. 2137-2139.
81. **Morosini M.I. *et al.***: Antibiotic coresistance in extended-spectrum-beta-lactamase-producing Enterobacteriaceae and in vitro activity of tigecycline. Antimicrob Agents Chemother, 2006. 50(8): p. 2695-2699.
82. **Datta N. and Kontomichalou P.**: Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nature, 1965. 208(5007): p. 239-241.
83. **Medeiros A.A.**: Beta-lactamases. Br Med Bull, 1984. 40(1): p. 18-27.
84. **Jacoby G.A. and Medeiros A.A.**: More extended-spectrum beta-lactamases. Antimicrob Agents Chemother, 1991. 35(9): p. 1697-1704.
85. **Paterson D.L. and Bonomo R.A.**: Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev, 2005. 18(4): p. 657-686.
86. **Sougakoff W. *et al.***: Plasmid-mediated resistance to third-generation cephalosporins caused by point mutations in TEM-type penicillinase genes. Rev Infect Dis, 1988. 10(4): p. 879-884.
87. **Oliver A. *et al.***: Nucleotide sequence and characterization of a novel cefotaxime-hydrolyzing beta-lactamase (CTX-M-10) isolated in Spain. Antimicrob Agents Chemother, 2001. 45(2): p. 616-620.
88. **Bradford P.A. *et al.***: CTX-M-5, a novel cefotaxime-hydrolyzing beta-lactamase from an outbreak of *Salmonella typhimurium* in Latvia. Antimicrob Agents Chemother, 1998. 42(8): p. 1980-1984.

89. **Canton R. and Coque T.M.**: The CTX-M beta-lactamase pandemic. Curr Opin Microbiol, 2006. 9(5): p. 466-475.
90. **Matsumoto Y. *et al.***: Novel plasmid-mediated beta-lactamase from *Escherichia coli* that inactivates oxyimino-cephalosporins. Antimicrob Agents Chemother, 1988. 32(8): p. 1243-1246.
91. **Bauernfeind A. *et al.***: A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. Infection, 1990. 18(5): p. 294-298.
92. **Power P. *et al.***: Cefotaxime-hydrolysing beta lactamases in *Morganella morganii*. Eur J Clin Microbiol Infect Dis, 1999. 18(10): p. 743-747.
93. **Barthelemy M. *et al.***: Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum beta-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca*. Biochim Biophys Acta, 1992. 1122(1): p. 15-22.
94. **Humeniuk C. *et al.***: Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob Agents Chemother, 2002. 46(9): p. 3045-3049.
95. **Poirel L. *et al.***: Chromosome-encoded Ambler class A beta-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. Antimicrob Agents Chemother, 2002. 46(12): p. 4038-4040.
96. **Weldhagen G.F. *et al.***: Ambler class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: Novel developments and clinical impact. Antimicrob Agents Chemother, 2003. 47(8): p. 2385-2392.
97. **Danel F. *et al.***: OXA-17, a further extended-spectrum variant of OXA-10 beta-lactamase, isolated from *Pseudomonas aeruginosa*. Antimicrob Agents Chemother, 1999. 43(6): p. 1362-1366.
98. **Hall L.M. *et al.***: OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) beta-lactamase from *Pseudomonas aeruginosa*. Antimicrob Agents Chemother, 1993. 37(8): p. 1637-1644.
99. **Danel F. *et al.***: OXA-14, another extended-spectrum variant of OXA-10 (PSE-2) beta-lactamase from *Pseudomonas aeruginosa*. Antimicrob Agents Chemother, 1995. 39(8): p. 1881-1884.
100. **Stolle I. *et al.***: Emergence of OXA-48 carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* in dogs. J Antimicrob Chemother, 2013. 68(12): p. 2802-2808.
101. **Walsh T.R.**: Emerging carbapenemases: a global perspective. Int J Antimicrob Agents, 2010. 36: p. 8-14.
102. **Lautenbach E. *et al.***: Epidemiological investigation of fluoroquinolone resistance in infections due to extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*. Clin Infect Dis, 2001. 33(8): p. 1288-1294.
103. **Studdert P.V. *et al.***: *Saunders Comprehensive Veterinary Dictionary*. Third edition ed. 2007, New York (USA): Elsevier.
104. **Boyce J.M. *et al.***: Meticillin-resistant *Staphylococcus aureus*. Lancet Infect Dis, 2005. 5(10): p. 653-663.
105. **CDC**: *Antibiotic Resistance Threats in the United States* 2013: Atlanta (USA).
106. **Navon-Venezia S. *et al.***: Update on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. Curr Opin Infect Dis, 2005. 18(4): p. 306-313.
107. **Brazier J.S.**: *Clostridium difficile*: from obscurity to superbug. Brit J Biomed Sci, 2008. 65(1): p. 39-44.
108. **Barriere S.L.**: Clinical, economic and societal impact of antibiotic resistance. Expert Opin Pharmaco, 2015. 16(2): p. 151-153.
109. **Tangden T. and Giske C.G.**: Global dissemination of extensively drug-resistant carbapenemase-producing Enterobacteriaceae: clinical perspectives on detection, treatment and infection control. J Intern Med, 2015. 277(5): p. 501-512.
110. **Tripathi P.C. *et al.***: Clinical and antimicrobial profile of *Acinetobacter spp*.: An emerging nosocomial superbug. Adv Biomed Res, 2014. 3: p. 13.
111. **Johnson J.R. and Russo T.A.**: Molecular epidemiology of extraintestinal pathogenic (uropathogenic) *Escherichia coli*. Int J Med Microbiol, 2005. 295(6-7): p. 383-404.
112. **Kaper J.B. *et al.***: Pathogenic *Escherichia coli*. Nat Rev Microbiol, 2004. 2(2): p. 123-140.

113. **Hahn H.**: *Medizinische Mikrobiologie und Infektiologie*. 2008, Berlin (Germany): Springer.
114. **Edberg S.C. *et al.***: *Escherichia coli*: the best biological drinking water indicator for public health protection. J Appl Microbiol, 2000. 88: p. 106-116.
115. **Marshall K.M. *et al.***: Identification of *Escherichia coli* O157:H7 meat processing indicators for fresh meat through comparison of the effects of selected antimicrobial interventions. J Food Protect, 2005. 68(12): p. 2580-2586.
116. **Reid G. *et al.***: Can bacterial interference prevent infection? Trends Microbiol, 2001. 9(9): p. 424-428.
117. **Hudault S. *et al.***: *Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection. Gut, 2001. 49(1): p. 47-55.
118. **Rogers B.A. *et al.***: *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. J Antimicrob Chemother, 2011. 66(1): p. 1-14.
119. **Circella E. *et al.***: Virulence-associated genes in avian pathogenic *Escherichia coli* from laying hens in Apulia, Southern Italy. Br Poult Sci, 2012. 53(4): p. 465-470.
120. **Kaase M. *et al.***: The German EHEC outbreak strain ST678 harbours the CTX-M-15 extended-spectrum beta-lactamase (ESBL). Int J Med Microbiol, 2011. 301: p. 18-18.
121. **Kitzis M.D. *et al.***: Dissemination of the novel plasmid-mediated beta-lactamase CTX-1, which confers resistance to broad-spectrum cephalosporins, and its inhibition by beta-lactamase inhibitors. Antimicrob Agents Chemother, 1988. 32(1): p. 9-14.
122. **Radice M. *et al.***: Early dissemination of CTX-M-derived enzymes in South America. Antimicrob Agents Chemother, 2002. 46(2): p. 602-604.
123. **Guzman-Blanco M. *et al.***: Extended spectrum beta-lactamase producers among nosocomial Enterobacteriaceae in Latin America. Braz J Infect Dis, 2014. 18(4): p. 421-433.
124. **Borg M.A. *et al.***: Antibiotic resistance in the southeastern Mediterranean--preliminary results from the ARMed project. Euro Surveill, 2006. 11(7): p. 164-167.
125. **Brinas L. *et al.***: Detection of CMY-2, CTX-M-14, and SHV-12 beta-lactamases in *Escherichia coli* fecal-sample isolates from healthy chickens. Antimicrob Agents Chemother, 2003. 47(6): p. 2056-2058.
126. **Kojima A. *et al.***: Extended-spectrum- beta -lactamase-producing *Escherichia coli* Strains Isolated from farm animals from 1999 to 2002: report from the Japanese Veterinary Antimicrobial Resistance Monitoring program. Antimicrob Agents Chemother, 2005. 49(8): p. 3533-3537.
127. **Duan R.S. *et al.***: *Escherichia coli* producing CTX-M beta-lactamases in food animals in Hong Kong. Microb Drug Resist, 2006. 12(2): p. 145-148.
128. **Sousa M. *et al.***: Gilthead seabream (*Sparus aurata*) as carriers of SHV-12 and TEM-52 extended-spectrum beta-lactamases-containing *Escherichia coli* isolates. Foodborne Pathog Dis, 2011. 8(10): p. 1139-1141.
129. **Wallensten A. *et al.***: Extended spectrum beta-lactamases detected in *Escherichia coli* from gulls in Stockholm, Sweden. Infect Ecol Epidemiol, 2011. 1: p. 10.3402/iee.v1i0.7030.
130. **Costa D. *et al.***: Detection of *Escherichia coli* harbouring extended-spectrum beta-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal. J Antimicrob Chemother, 2006. 58(6): p. 1311-1312.
131. **Poeta P. *et al.***: Seagulls of the Berlengas natural reserve of Portugal as carriers of fecal *Escherichia coli* harboring CTX-M and TEM extended-spectrum beta-lactamases. Appl Environ Microbiol, 2008. 74(23): p. 7439-7441.
132. **Literak I. *et al.***: Antimicrobial-resistant faecal *Escherichia coli* in wild mammals in central Europe: multiresistant *Escherichia coli* producing extended-spectrum beta-lactamases in wild boars. J Appl Microbiol, 2010. 108(5): p. 1702-1711.
133. **Bonnedahl J. *et al.***: Dissemination of *Escherichia coli* with CTX-M type ESBL between humans and yellow-legged gulls in the south of France. Plos One, 2009. 4(6): p. e5958.
134. **Pinto L. *et al.***: Genetic detection of extended-spectrum beta-lactamase-containing *Escherichia coli* isolates from birds of prey from Serra da Estrela natural reserve in Portugal. Appl Environ Microbiol, 2010. 76(12): p. 4118-4120.

135. **Radhouani H. *et al.***: Detection of *Escherichia coli* harbouring extended-spectrum beta-lactamases of the CTX-M classes in faecal samples of common buzzards (*Buteo buteo*). J Antimicrob Chemother, 2010. 65(1): p. 171-173.
136. **Skurnik D. *et al.***: Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. J Antimicrob Chemother, 2006. 57(6): p. 1215-1219.
137. **Allen H.K. *et al.***: Call of the wild: antibiotic resistance genes in natural environments. Nat Rev Microbiol, 2010. 8(4): p. 251-259.
138. **Hernandez J. *et al.***: Globally disseminated human pathogenic *Escherichia coli* of O25b-ST131 clone, harbouring bla(CTX-M-15), found in Glaucous-winged gull at remote Commander Islands, Russia. Env Microbiol Rep, 2010. 2(2): p. 329-332.
139. **Gordon D.M. and Cowling A.**: The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. Microbiology, 2003. 149(Pt 12): p. 3575-3586.
140. **Guenther S. *et al.***: Detection of pandemic B2-O25-ST131 *Escherichia coli* harbouring the CTX-M-9 extended-spectrum beta-lactamase type in a feral urban brown rat (*Rattus norvegicus*). J Antimicrob Chemother, 2010. 65(3): p. 582-584.
141. **Blaak H. *et al.***: Detection of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* on flies at poultry farms. Appl Environ Microbiol, 2014. 80(1): p. 239-246.
142. **Stalder G.L. *et al.***: Diversity of enterobacteria including beta-lactamase producing isolates associated with the Spanish slug (*Arion vulgaris*). Sci Total Environ, 2014. 479-480: p. 11-16.
143. **Smet A. *et al.***: Broad-spectrum beta-lactamases among Enterobacteriaceae of animal origin: molecular aspects, mobility and impact on public health. FEMS Microbiol Rev, 2010. 34(3): p. 295-316.
144. **Reinthaler F.F. *et al.***: ESBL-producing *E. coli* in Austrian sewage sludge. Water Res, 2010. 44(6): p. 1981-1985.
145. **Zarfel G. *et al.***: Comparison of extended-spectrum-beta-lactamase (ESBL) carrying *Escherichia coli* from sewage sludge and human urinary tract infection. Environ Pollut, 2013. 173: p. 192-199.
146. **Dhanji H. *et al.***: Isolation of fluoroquinolone-resistant O25b:H4-ST131 *Escherichia coli* with CTX-M-14 extended-spectrum beta-lactamase from UK river water. J Antimicrob Chemother, 2011. 66(3): p. 512-516.
147. **Zhang X.X. *et al.***: Antibiotic resistance genes in water environment. Appl Microbiol Biotechnol, 2009. 82(3): p. 397-414.
148. **Procter T.D. *et al.***: A cross-sectional study examining the prevalence and risk factors for anti-microbial-resistant generic *Escherichia coli* in domestic dogs that frequent dog parks in three cities in South-Western Ontario, Canada. Zoonoses Public Hlth, 2014. 61(4): p. 250-259.
149. **Cinquepalmi V. *et al.***: Environmental contamination by dogs faeces: a public health problem? Int J Environ Res Public Health, 2013. 10(1): p. 72-84.
150. **Ewers C. *et al.***: Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-beta-lactamase-producing *Escherichia coli* among companion animals. J Antimicrob Chemother, 2010. 65: p. 651-660.
151. **Murphy C. *et al.***: Occurrence of antimicrobial resistant bacteria in healthy dogs and cats presented to private veterinary hospitals in southern Ontario: A preliminary study. Can Vet J, 2009. 50(10): p. 1047-1053.
152. **Green H.C. *et al.***: Development of rapid canine fecal source identification PCR-based assays. Environ Sci Technol, 2014. 48(19): p. 11453-11461.
153. **Ervin J.S. *et al.***: Microbial source tracking in a coastal California watershed reveals canines as controllable sources of fecal contamination. Environ Sci Technol, 2014. 48(16): p. 9043-9052.
154. **Patterson E.L. *et al.***: Isolation of cephalosporin C. J Med Chem, 1964. 7: p. 689.
155. **Balant L. *et al.***: Clinical pharmacokinetics of the third generation cephalosporins. Clin Pharmacokinet, 1985. 10(2): p. 101-143.
156. **Auckenthaler R. and Waldvogel F.A.**: How many cephalosporins does the clinician need? Schweiz Med Wochenschr, 1981. 111(42): p. 1554-1556.

157. **Shiraki Y. *et al.***: *Escherichia coli* producing CTX-M-2 beta-lactamase in cattle, Japan. Emerg Infect Dis, 2004. 10(1): p. 69-75.
158. **Mosby**: *Mosby's Medical Dictionary* 2009, New York (USA): Elsevier.
159. **Hartmann A. *et al.***: Occurrence of CTX-M producing *Escherichia coli* in soils, cattle, and farm environment in France (Burgundy Region). Front Microbiol, 2012. 3: p. 83.
160. **Ruimy R. *et al.***: Organic and conventional fruits and vegetables contain equivalent counts of Gram-negative bacteria expressing resistance to antibacterial agents. Environ Microbiol, 2010. 12(3): p. 608-615.
161. **Ewers C. *et al.***: Extended-spectrum beta-lactamases-producing Gram-negative bacteria in companion animals: action is clearly warranted! Berl Munch Tierarztl Wochenschr, 2011. 124(3): p. 94-101.
162. **Wieler L.H. *et al.***: Methicillin-resistant staphylococci (MRS) and extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae in companion animals: nosocomial infections as one reason for the rising prevalence of these potential zoonotic pathogens in clinical samples. Int J Med Microbiol, 2011. 301(8): p. 635-41.
163. **Blouin D. and Bloomington I.**: *All in the family? Understanding the meaning of dogs and cats in the lives of American pet owners*. 2008, Indiana (USA): Thesis Indiana University.
164. **Walther B. *et al.***: Sharing more than friendship - nasal colonization with coagulase-positive *Staphylococci* (CPS) and co-habitation aspects of dogs and their owners. Plos One, 2012. 7(4): p. e35197.
165. **Song S.J. *et al.***: Cohabiting family members share microbiota with one another and with their dogs. Elife, 2013. 2: p. e00458.
166. **Hayward M.**: *Risks of zoonoses from dogs on sporting fields*. Australian Veterinary Association, Centre for Companion Animals in the Community. 2004, St Leonards (Australia).
167. **Ahmed L.N. *et al.***: An exploratory study of dog park visits as a risk factor for exposure to drug-resistant extra-intestinal pathogenic *E. coli* (ExPEC). BMC Res Notes, 2015. 8: p. 137.
168. **Ghosh A. *et al.***: Resident cats in small animal veterinary hospitals carry multi-drug resistant enterococci and are likely involved cross-contamination of the hospital environment. Front Microbiol, 2012. 3: p. 62.
169. **Guenther S. *et al.***: Is fecal carriage of extended-spectrum-beta-lactamase-producing *Escherichia coli* in uban rats a risk for public health? Antimicrob Agents Chemother, 2013. 57(5): p. 2424-2425.
170. **Yang C.M. *et al.***: Comparison of antimicrobial resistance patterns between clinical and sewage isolates in a regional hospital in Taiwan. Lett Appl Microbiol, 2009. 48(5): p. 560-565.
171. **Salyers A.A.**: An overview of the genetic basis of antibiotic resistance in bacteria and its implications for agriculture. Anim Biotechnol, 2002. 13(1): p. 1-5.
172. **Minarini L.A. *et al.***: Multilocus sequence typing of uropathogenic ESBL-producing *Escherichia coli* isolated in a Brazilian community. Curr Microbiol, 2007. 55(6): p. 524-529.
173. **Blanco M. *et al.***: Molecular epidemiology of *Escherichia coli* producing extended-spectrum beta-lactamases in Lugo (Spain): dissemination of clone O25b:H4-ST131 producing CTX-M-15. J Antimicrob Chemother, 2009. 63(6): p. 1135-1141.
174. **Naseer U. *et al.***: Molecular characterization of CTX-M-15-producing clinical isolates of *Escherichia coli* reveals the spread of multidrug-resistant ST131 (O25:H4) and ST964 (O102:H6) strains in Norway. APMIS, 2009. 117(7): p. 526-536.
175. **Oteo J. *et al.***: Extended-spectrum beta-lactamase-producing *Escherichia coli* in Spain belong to a large variety of multilocus sequence typing types, including ST10 complex/A, ST23 complex/A and ST131/B2. Int J Antimicrob Agents, 2009. 34(2): p. 173-176.
176. **Sidjabat H.E. *et al.***: Molecular epidemiology of CTX-M-producing *Escherichia coli* isolates at a tertiary medical center in western Pennsylvania. Antimicrob Agents Chemother, 2009. 53(11): p. 4733-4739.
177. **Doi Y. *et al.***: Extended-spectrum and CMY-type beta-lactamase-producing *Escherichia coli* in clinical samples and retail meat from Pittsburgh, USA and Seville, Spain. Clin Microbiol Infect, 2010. 16(1): p. 33-38.

178. **Platell J.L. *et al.***: Multidrug-resistant extraintestinal pathogenic *Escherichia coli* of sequence type ST131 in animals and foods. Vet Microbiol, 2011. 153(1-2): p. 99-108.
179. **Ewers C. *et al.***: Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum- beta -lactamase-producing *Escherichia coli* among companion animals. Journal of Antimicrobial Chemotherapy, 2010. 65(4): p. 651-660.
180. **Achtman M.**: *A Phylogenetic Perspective on Molecular Epidemiology*, in *Molecular Medical Microbiology*. 2001, Academic Press: London (UK).
181. **Croxatto A. *et al.***: Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS Microbiol Rev, 2012. 36(2): p. 380-407.
182. **Bertelli C. and Greub G.**: Rapid bacterial genome sequencing: methods and applications in clinical microbiology. Clin Microbiol Infect, 2013. 19(9): p. 803-813.
183. **Chaudhuri R.R. and Henderson I.R.**: The evolution of the *Escherichia coli* phylogeny. Infect Genet Evol, 2012. 12(2): p. 214-226.
184. **Sabat A.J. *et al.***: Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Euro Surveill 2013. 18(4): p. 17-30.
185. **Maatallah M. *et al.***: Four genotyping schemes for phylogenetic analysis of *Pseudomonas aeruginosa*: comparison of their congruence with multi-locus sequence typing. PLoS One, 2013. 8(12): p. e82069.
186. **Foley S.L. *et al.***: Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. Infect Genet Evol, 2009. 9(4): p. 430-440.
187. **Maiden M.C. *et al.***: Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA, 1998. 95(6): p. 3140-3145.
188. **Feil E.J. *et al.***: eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol, 2004. 186(5): p. 1518-1530.
189. **Nemoy L.L. *et al.***: Multilocus sequence typing versus pulsed-field gel electrophoresis for characterization of extended-spectrum Beta-lactamase-producing *Escherichia coli* isolates. J Clin Microbiol, 2005. 43(4): p. 1776-1781.
190. **Foley S.L. *et al.***: Evaluation of molecular typing methods for *Escherichia coli* O157:H7 isolates from cattle, food, and humans. J Food Protect, 2004. 67(4): p. 651-657.
191. **Johnson J.R. and Stell A.L.**: Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis, 2000. 181(1): p. 261-272.
192. **Picard B. *et al.***: The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infect Immun, 1999. 67(2): p. 546-553.
193. **Bingen E. *et al.***: Phylogenetic analysis of *Escherichia coli* strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains. J Infect Dis, 1998. 177(3): p. 642-650.
194. **Lecointre G. *et al.***: *Escherichia coli* molecular phylogeny using the incongruence length difference test. Mol Biol Evol, 1998. 15(12): p. 1685-1695.
195. **Ochman H. and Selander R.K.**: Standard reference strains of *Escherichia coli* from natural populations. J Bacteriol, 1984. 157(2): p. 690-693.
196. **Desjardins P. *et al.***: Sex in *Escherichia coli* does not disrupt the clonal structure of the population - evidence from random amplified polymorphic DNA and restriction-fragment-length-polymorphism. J Mol Evol, 1995. 41(4): p. 440-448.
197. **Clermont O. *et al.***: Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol, 2000. 66(10): p. 4555-4558.
198. **Tenaillon O. *et al.***: The population genetics of commensal *Escherichia coli*. Nat Rev Microbiol, 2010. 8(3): p. 207-217.
199. **Wirth T. *et al.***: Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol, 2006. 60(5): p. 1136-1151.

200. **Tenover F.C. *et al.***: Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel-electrophoresis - criteria for bacterial strain typing. J Clin Microbiol, 1995. 33(9): p. 2233-2239.
201. **Karaolis D.K. *et al.***: Molecular evolution of the seventh-pandemic clone of *Vibrio cholerae* and its relationship to other pandemic and epidemic *V. cholerae* isolates. J Bacteriol, 1994. 176(20): p. 6199-6206.
202. **Popovic T. *et al.***: Epidemiologic application of a standardized ribotype scheme for *Vibrio cholerae* O1. J Clin Microbiol, 1993. 31(9): p. 2474-2482.
203. **Gerner-Smidt P. and Scheutz F.**: Standardized pulsed-field gel electrophoresis of Shiga toxin-producing *Escherichia coli*: The PulseNet Europe feasibility study. Foodborne Pathog Dis, 2006. 3(1): p. 74-80.
204. **Finney M.**: Pulsed-field gel electrophoresis. Curr Protoc Mol Biol, 2001. 2: p. Unit 2.5B.
205. **Goering R.V.**: Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. Infect Genet Evol, 2010. 10(7): p. 866-875.
206. **Fournier P.E. *et al.***: Bacterial genome sequencing and its use in infectious diseases. Lancet Infect Dis, 2007. 7(11): p. 711-723.
207. **Grad Y.H. *et al.***: Genomic epidemiology of the *Escherichia coli* O104:H4 outbreaks in Europe, 2011 Prot Natl Acad Sci USA, 2012. 109(14): p. 5547-5547.
208. **Mellmann A. *et al.***: Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. PLoS One, 2011. 6(7): p. e22751.
209. **Fleischmann R.D. *et al.***: Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science, 1995. 269(5223): p. 496-512.
210. **Sanger F. *et al.***: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA, 1977. 74(12): p. 5463-7.
211. **Sanger F. and Coulson A.R.**: A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol, 1975. 94(3): p. 441-8.
212. **Metzker M.L.**: Emerging technologies in DNA sequencing. Genome Res, 2005. 15(12): p. 1767-1776.
213. **Loman N.J. *et al.***: High-throughput bacterial genome sequencing: an embarrassment of choice, a world of opportunity. Nat Rev Microbiol, 2012. 10(9): p. 599-606.
214. **Cebula T.A. *et al.***: Molecular applications for identifying microbial pathogens in the post-9/11 era. Expert Rev Mol Diagn, 2005. 5(3): p. 431-445.
215. **Le V.T.M. and Diep B.A.**: Selected insights from application of whole-genome sequencing for outbreak investigations. Curr Opin Crit Care, 2013. 19(5): p. 432-439.
216. **Touchon M. *et al.***: Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. Plos Genet, 2009. 5(1): p. e1000344.
217. **Silva N. *et al.***: Molecular characterization of vancomycin-resistant enterococci and extended-spectrum beta-lactamase-containing *Escherichia coli* isolates in wild birds from the Azores Archipelago. Avian Pathol, 2011. 40(5): p. 473-479.
218. **Hasan B. *et al.***: Dissemination of multidrug resistant ESBL-producing *E. coli* O25b-ST131 clone and role of House Crow (*Corvus splendens*) foraging on hospital waste in Bangladesh. Clin Microbiol Infect, 2015. 21(11): p. 1000.e1-4.
219. **Harada K. *et al.***: Clonal spread of antimicrobial-resistant *Escherichia coli* isolates among pups in two kennels. Acta Vet Scand, 2011. 53: p. 11.
220. **Albrechtova K. *et al.***: Dogs of nomadic pastoralists in Northern Kenya are reservoirs of plasmid-mediated cephalosporin- and quinolone-resistant *Escherichia coli*, including pandemic clone B2-O25-ST131. Antimicrob Agents Chemother, 2012. 56(7): p. 4013-4017.
221. **Ewers C.**: *Zoonoses- Infections affecting humans and animals*, A. Sing, Editor. 2014, Springer: Heidelberg (Germany).
222. **Wieler L.H. *et al.***: No evidence of the Shiga toxin-producing *E. coli* O104:H4 outbreak strain or enteroaggregative *E. coli* (EAEC) found in cattle faeces in northern Germany, the hotspot of the 2011 HUS outbreak area. Gut Pathog, 2011. 3: p. 17.

223. **Watson E. *et al.***: Epidemiology of extended spectrum beta-lactamase *E-coli* (CTX-M-15) on a commercial dairy farm. Vet Microbiol, 2012. 154(3-4): p. 339-346.
224. **SWEDRES-SVARM**: *Consumption of antibiotics and occurrence of antibiotic resistance in Sweden*. 2014, Solna/Uppsala (Sweden).
225. **Johnson J.R. *et al.***: Sharing of *Escherichia coli* sequence sype ST131 and other multidrug-resistant and urovirulent *E. coli* strains among dogs and cats within a household. J Clin Microbiol, 2009. 47(11): p. 3721-3725.
226. **Mavroidi A. *et al.***: Emergence of *Escherichia coli* sequence type 410 (ST410) with KPC-2 beta-lactamase. Int J Antimicrob Agents, 2012. 39(3): p. 247-250.
227. **Giufre M. *et al.***: Whole-genome sequences of multidrug-resistant *Escherichia coli* strains sharing the same sequence type (ST410) and isolated from human and avian sources in Italy. Genome Announc, 2015. 3(4): p. e00757-15.
228. **Rocha-Gracia R.C. *et al.***: Faecal *Escherichia coli* isolates from healthy dogs harbour CTX-M-15 and CMY-2 beta-lactamases. Vet J, 2015. 203(3): p. 315-319.
229. **Xu G. *et al.***: Emergence of KPC-2-producing *Escherichia coli* isolates in an urban river in Harbin, China. World J Microbiol Biotechnol, 2015. 31(9): p. 1443-50.
230. **Johnson J.R. *et al.***: Epidemiological correlates of virulence genotype and phylogenetic background among *Escherichia coli* blood isolates from adults with diverse-source bacteremia. J Infect Dis, 2002. 185(10): p. 1439-1447.
231. **Carrico J.A. *et al.***: Assessment of band-based similarity coefficients for automatic type and subtype classification of microbial isolates analyzed by pulsed-field gel electrophoresis. J Clin Microbiol, 2005. 43(11): p. 5483-5490.
232. **Department of Health**: *UK Five Year Antimicrobial Resistance Strategy 2013 to 2018*. 2013, London (UK).
233. **Schaffert R.M. and Strauch D.**: Naturally infected dog droppings from public parks and playgrounds as a possible source of infections with *Salmonellae* and helminths. Ann Ist Super Sanita, 1978. 14(2): p. 295-300.
234. **Badura A. *et al.***: Prevalence, antibiotic resistance patterns and molecular characterization of *Escherichia coli* from Austrian sandpits. Environ Pollut, 2014. 194: p. 24-30.
235. **WHO**: *Integrated Disease Surveillance Programme*. 2015, New York (USA).

9. LIST OF PUBLICATIONS

Research articles

- **Katharina Schaufler**, Torsten Semmler, Derek J. Pickard, María de Toro, Fernando de la Cruz, Lothar H. Wieler, Christa Ewers and Sebastian Guenther: Carriage of extended-spectrum beta-lactamase-plasmids does not reduce fitness but enhances virulence in some strains of pandemic *E. coli* lineages (2016). Frontiers in Microbiology 7: 336
- **Katharina Schaufler**, Torsten Semmler, Lothar H. Wieler, Michael Wöhrmann, Ramani Baddam, Niyaz Ahmed, Kerstin Müller, Axel Kola, Angelika Fruth, Christa Ewers and Sebastian Guenther: Clonal spread and interspecies transmission of clinically relevant ESBL-producing *Escherichia coli* of ST410 – another successful pandemic clone (2015)? FEMS Microbiology Ecology 92(1): fiv155
- **Katharina Schaufler**, Astrid Bethe, Antina Lübke-Becker, Christa Ewers, Barbara Kohn, Lothar H. Wieler and Sebastian Guenther: Putative connection between zoonotic multiresistant extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in dog feces from a veterinary campus and clinical isolates from dogs (2015). Infection Ecology and Epidemiology 5: 25334
- **Katharina Schaufler**, Lothar H. Wieler, Torsten Semmler, Christa Ewers and Sebastian Guenther: ESBL-plasmids carrying toxin-antitoxin systems can be “cured” of wild-type *Escherichia coli* using a heat technique (2013). Gut Pathogens 5(1): 34
- Stefan Rödiger, Toni Kramer, Ulrike Frömmel, Jörg Weinreich, Dirk Roggenbuck, Sebastian Guenther, **Katharina Schaufler,** Christian Schröder and Peter Schierack: Intestinal *Escherichia coli* colonization in a mallard duck population over four consecutive winter seasons (2015). Environmental Microbiology 17(9): 3352-61
- Christa Ewers, Astrid Bethe, Ivonne Stamm, Mirjam Grobbel, Peter A. Kopp, Beatriz Guerra, Michael Stubbe, Yohei Doi, Zhiyong Zong, Axel Kola, **Katharina Schaufler**, Torsten Semmler, Angelika Fruth, Lothar H. Wieler and Sebastian Guenther: CTX-M-15-D-ST648 *Escherichia coli* from companion animals and horses: another clone combining multi-resistance and extraintestinal virulence (2014)? Journal of Antimicrobial Chemotherapy 69(5): 1224-30
- Sebastian Guenther, Julia Wuttke, Astrid Bethe, Jiří Vojtěch, **Katharina Schaufler**, Torsten Semmler, Rainer G. Ulrich, Lothar H. Wieler and Christa Ewers: Fecal carriage of extended-spectrum beta-lactamase-producing *E. coli* in urban rats, a risk for public health (2013)? Antimicrobial Agents and Chemotherapy 57(5): 2424-25

Poster presentations

- **Katharina Schaufler**, Michael Wöhrmann, Ramani Baddam, Kerstin Müller, Petra Gastmeier, Barbara Kohn, Torsten Semmler, Lothar H. Wieler, Christa Ewers and Sebastian Guenther: Whole-genome analysis of ESBL-producing *E. coli* of ST410 reveals interspecies transmissions of identical bacterial clones between avian wildlife, humans, companion animals and the environment, Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Münster, Germany, 2015
- **Katharina Schaufler**, Torsten Semmler, Lothar H. Wieler, Christa Ewers, Derek J. Pickard, Gordon Dougan and Sebastian Guenther: ESBL-plasmids influence the chromosomally-encoded *csgD*-pathway in multi-resistant ST131 and ST648 *E. coli*, possibly contributing to their pandemic success in numerous habitats, Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Münster, Germany, 2015
- **Katharina Schaufler**, Christa Ewers, Torsten Semmler, Derek J. Pickard, Gordon Dougan, Lothar H. Wieler and Sebastian Guenther: ESBL-plasmids affect chromosomally encoded features in *Escherichia coli* of pandemic sequence types ST131 and ST648, National Symposium on Zoonosis Research, Berlin, Germany, 2014
- **Katharina Schaufler**, Christa Ewers, Torsten Semmler, Lothar H. Wieler and Sebastian Guenther: ESBL-plasmids affect features beyond resistance in *Escherichia coli* of pandemic sequence types ST131 and ST648, Interscience Conference of Antimicrobial Agents and Chemotherapy, Washington D.C., U.S.A., 2014
- **Katharina Schaufler**, Astrid Bethe, Antina Lübke-Becker, Christa Ewers, Barbara Kohn, Lothar H. Wieler and Sebastian Guenther: Urban dog faeces: a possible infection source for ESBL-producing *E. coli*? Fachgruppentagung der Deutschen Veterinärmedizinischen Gesellschaft Bakteriologie/Mykologie, Freising, Germany, 2014
- **Katharina Schaufler**, Christa Ewers, Torsten Semmler, Derek J. Pickard, Gordon Dougan, Lothar H. Wieler and Sebastian Guenther: Influence of ESBL-plasmids on features other than resistance in *Escherichia coli* of pandemic sequence types ST131 & ST648, Fachgruppentagung der Deutschen Veterinärmedizinischen Gesellschaft Bakteriologie/Mykologie, Germany, 2014
- **Katharina Schaufler**, Torsten Semmler, Lothar H. Wieler, Christa Ewers and Sebastian Guenther: Phenotypic comparison of wildtype ESBL-*E. coli* of the pandemic sequence types ST131 and ST648 to their ESBL-plasmid-cured variants, Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Rostock, Germany, 2013

- **Katharina Schaufler**, Torsten Semmler, Lothar H. Wieler, Christa Ewers and Sebastian Guenther: A pheno- and genotypic comparison of wildtype ESBL- *E. coli* strains to their ESBL-plasmid-cured variants (PCV), Congress of Federation of European Microbiological Societies, Leipzig, Germany, 2013
- **Katharina Schaufler**, Torsten Semmler, Lothar H. Wieler, Christa Ewers and Sebastian Guenther: Comparative analysis of wildtype ESBL- *E. coli* strains and their plasmid-cured variants (PCV), IV. EHEC-Workshop, Wildbad Kreuth, Germany, 2013

10. DANKSAGUNG

Ich danke meinem Doktorvater Herrn Prof. Dr. Lothar H. Wieler für die Möglichkeit, am Institut für Mikrobiologie und Tierseuchen zu promovieren. Sie hatten auch nach Ihrem Wechsel zum Robert Koch Institut und trotz vieler neuer und zeitintensiver Aufgaben immer ein offenes Ohr, hilfreiche Ratschläge und sehr viel Verständnis. Herzlichen Dank für die sehr gute Betreuung.

Herrn Priv.-Doz. Dr. Sebastian Günther möchte ich für die Überlassung des Großteils dieser Projektidee danken. Du warst an der Entwicklung dieser Arbeit, ob im Labor oder in der Theorie, maßgeblich beteiligt und ich konnte mich in allen Phasen immer auf dich verlassen. Vielen Dank für die Beantwortung meiner unzähligen Fragen, insbesondere während meiner Anfangszeit. Ohne dich wäre diese Dissertation nicht möglich gewesen.

Frau Prof. Dr. Christa Ewers danke ich für die erfolgreiche Zusammenarbeit in mehreren Projekten und für die hilfreichen Tipps und Ratschläge, auch im Rahmen dieser Doktorarbeit.

Ich danke der SONNENFELD-STIFTUNG und der Deutschen Forschungsgemeinschaft für die finanzielle Unterstützung.

Vielen Dank an die lieben Kolleginnen und Kollegen vom IMT, ihr habt mir meine Promotionszeit in Berlin und am Institut unvergesslich gemacht. Ich bedanke mich für die fachliche und moralische Unterstützung.

Insbesondere möchte ich meinen Freunden und meiner Familie danken. Ich liebe euch und ich hätte es ohne eure Unterstützung, euren Glauben an mich und an meine Fähigkeiten niemals geschafft und dafür bin ich sehr dankbar. Ihr habt mir auch in schwierigen Zeiten die nötige Kraft gegeben um weiterzumachen und mich daran erinnert, dass es ein Leben außerhalb der Arbeit gibt. Danke!

11. SELBSTSTÄNDIGKEITSERKLÄRUNG

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

Katharina Schaufler

Berlin, 13.06.2016