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Vergleichende Charakterisierung von ESBL-bildenden *Escherichia coli* in Diensthunden der Deutschen Bundeswehr

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3-APB	3-Amino-Phenyl-Borat
AmpC	ampicillin resistant gene C beta-lactamase
C/C	cefotaxime + clavulanic acid
CAZ	ceftazidime
CDC	Centre for Disease Control and Prevention
CDS	coding sequence
CEP	cefepime
CLSI	Clinical & Laboratory Standards Institute
CMC	cefepime + clavulanic acid
COX	cefoxitin
CTB	cefotaxime + 3-APB
CTX	cefotaxime
CTX-M	cefotaximase-Munich beta-lactamase
CZB	ceftazidime + 3-APB
CZC	ceftazidime + clavulanic acid
DART	Deutsche Antibiotika-Resistenzstrategie
E. coli	*Escherichia coli*
ECDC	European Centre for Disease Prevention and Control
ERT	ertapenem
ESBL	extended-spectrum beta-lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GC	growth control
GC/B	growth control + 3-APB
GC/E	growth control + EDTA
I	intermediate

MEB	meropenem + 3-APB
MEE	meropenem + EDTA
MER	meropenem
mg/L	milligram per litre
MHK	minimale Hemmkonzentration
MIC	minimal inhibitory concentration
MLST	multilocus sequence typing
n	number of isolates
na	not assigned
NMD-1	new-Delhi metallo-beta-lactamase 1
OXA	oxacillin-hydrolyzing beta-lactamase
R	resistant
S	susceptible
SHV	sulfhydryl variable beta-lactamase
SNP	single nucleotide polymorphism
ST	sequence type
TEM	temoneira beta-lactamase
VetCAST	Veterinary Antimicrobial Susceptibility Testing
VIM	verona integron-encoded metallo-beta-lactamase
WHO	World Health Organisation

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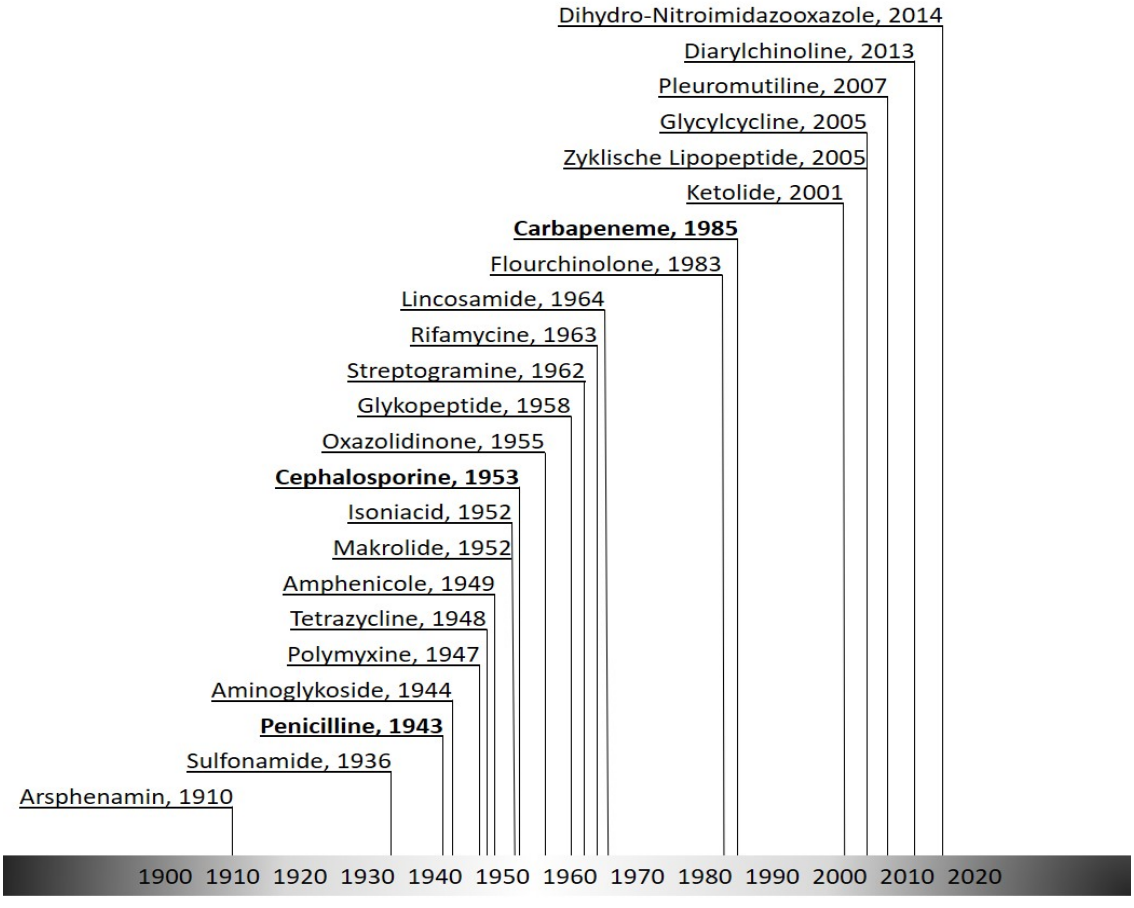
I. Introduction

Bacteria are one of the earliest forms of life on earth and ever since they had to defense their territory against other microorganisms, fungi and further competitors [1]. Therefore, microbial resistance is probably as old as bacteria themselves [2].

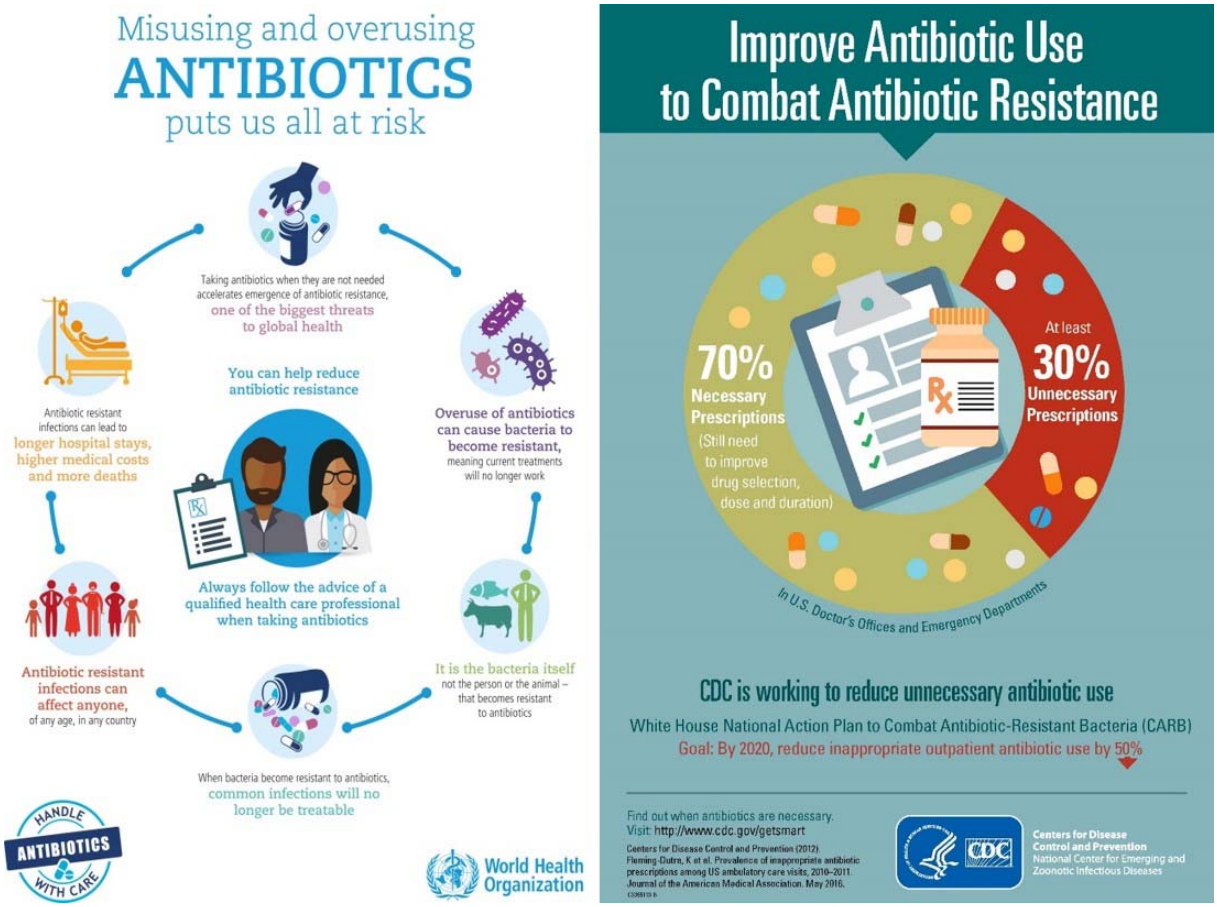
The microbiologist Robert Koch was one of the first who discovered the infectious character of microorganisms, and in 1884 he, Friedrich Loeffler and others set up the Koch's postulates for the characteristics and identification of infectious microorganisms [3]. In 1929, Alexander Fleming made the scientific discovery and found an agent of antimicrobial effect produced by *Penicillium notatum* [4-5]. Florey and Chain developed a method to extract the antibiotic penicillin out of *Penicillium* cultures that enabled the usage of this remedy during World War II [5]. Since then, the fast progressing development of various and inexpensive antibiotic substances for the use of human and animal medication shaped the Industrial Age (Fig. 1).

Figure 1: Discovery and year of introduction regarding new antibiotic substances with the beta-lactam antibiotics in bold.



By then it was not to be seen yet that abundant use of antibiotic substances increases the selection of antimicrobial resistant bacteria almost at the same time [2]. In animal husbandry, antibiotics were overprescribed and (ab)used to boost profit in the meat production business [6]. Human medicine faces the same problems of unchecked (mis)use of antimicrobial substances enabling the selection of hazardous resistances (Fig. 2). Broad cognition that antimicrobial resistance will pose a major problem in medicine alerted different committees such as the World Health Organization (WHO) and the European Centre for Disease Prevention and Control (ECDC) [7]. As consequence, public health authorities such as the WHO and the American Centers for Disease Control and Prevention (CDC) publicly warned about the misusage of antimicrobial substances and even published posters to arise interest in the society to this impending problem (Fig. 2).

Figure 2: Publicly published warnings regarding the misuse of antimicrobial substances.



Currently, in the twenty-first century, it is a mainline challenge in medicine to handle the tightrope walk between therapy using antibiotics and not using these to safe resources [8]. Regardless of the affected discipline such as human or veterinary medicine, joint efforts need to be done based on the one health approach and to set a trend against antimicrobial resistance [9]. The present work was done with the intention to investigate the prevalence of ESBL-producing bacteria in military work dogs. A major task was to make a rough estimate of the risk for special professional groups such as dog handlers and veterinarians.

Using a longitudinal timeline, the persistence and dynamic of these bacteria should be determined. Finally, and to compare the results at some epidemiological context, individual isolates from dogs were recovered originating from military area of operations.

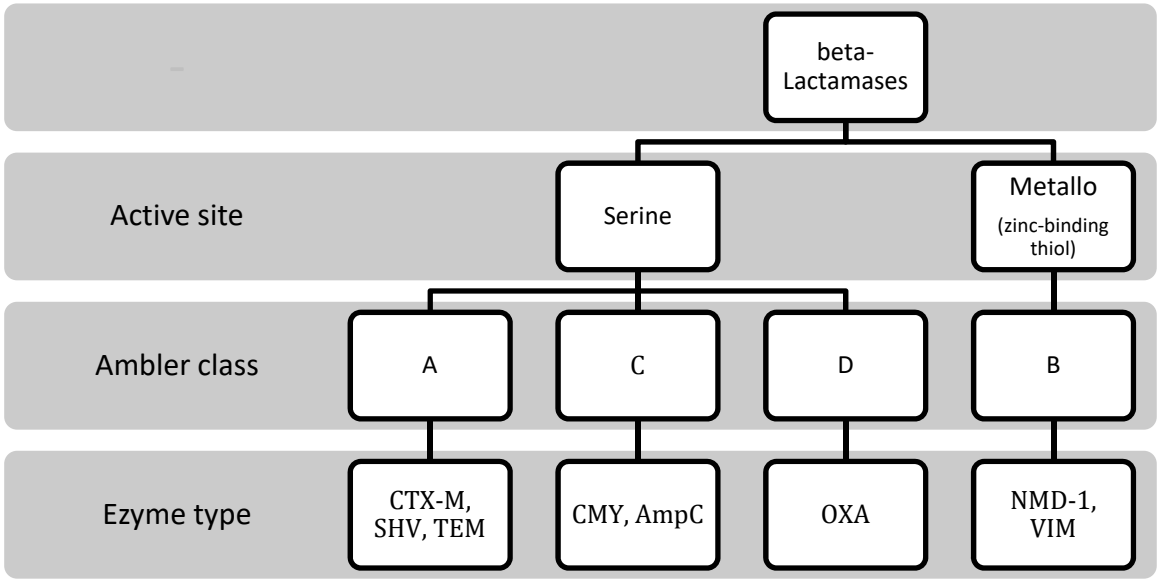
II. Literature review

1 Extended-spectrum beta-lactamases producing bacteria

Extended-spectrum beta-lactamases (ESBL) producers is a term for a subgroup of gram-negative bacteria, characterized by their enzymatic activity being able to destroy beta-lactam antibiotics with extended-spectrum such as 3rd and 4th generation cephalosporins [10]. Extended-spectrum beta-lactamases can be categorized by their molecular structure, used in the classification system by Ambler in 1980 (Fig. 3). Additionally to the Ambler classification scheme a further major scheme is the Bush-Jacoby-Medieros classification scheme categorizing extended-spectrum beta-lactamases by their functionality (Table 1) [10, 12].

Initially only plasmid-encoded beta-lactamases enabled to hydrolyse 3rd and 4th generation cephalosporins belonging to Ambler class A and D were signed as ESBL-producers [11]. A newer definition expands the term ESBL by Ambler class C beta-lactamases and carbapenemases (Fig. 3).

Figure 3: Ambler classification of beta-lactamases, adapted from [11].



However there is no standardized precise definition for the term ESBL in view of the fact that there are several schemes.

Table 1: Bush-Jacoby-Medeiros classification scheme for beta-lactamases, adapted from [13].

Group	Enzyme type	Inhibition by clavulanic acid	Molecular class	No. of enzymes	Example
1	Cephalosporinase	No	C	53	*E. cloacae* P99, MIR-1
2a	Penicillinase	Yes	A	20	*S. aureus*, *S. epidermidis*
2b	Broad-spectrum	Yes	A	16	TEM-1
2be	Extended-spectrum	Yes	A	38	TEM-3, SHV-2
2br	Inhibitor-resistant	Diminished	A	9	TRC-1
2c	Carbenicillinase	Yes	A	15	PSE-1, BRO-1
2d	Cloxacillinase	Yes	D or A	18	OXA-1, *Streptomyces cacaoi*
2e	Cephalosporinase	Yes	A	19	*Proteus vulgaris*
2f	Carbapenemase	Yes	A	3	*E. cloacae* IMI-1
3	Metalloenzyme	No	B	15	*Stenotrophomonas maltophilia* L1
4	Penicillinase	No		7	*Burkholderia cepacia*

Both schemes for the classification of beta-lactamases are useful for different aims. A precise definition for ESBL is not given in any of them [12]. Other systems focus on the epidemiological and clinical evaluation of bacterial resistance. For the mers of the present study we used standard guidelines published by the Clinical & Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for the classification of the isolates [15, 17-18, 20].

2 Beta-lactam antibiotics

The term beta-lactam originates from the chemical structure, a beta-lactam ring. The group of beta-lactam antibiotics comprises in addition to the primal substance penicillin and its derivatives the categories of cephalosporins, carbapenems and monobactams. The mechanism of action of all beta-lactam antibiotics is primary bactericidal, meaning that bacteria are damaged by the antibiotics and their proliferative growth is inhibited [18]. The monobactams are playing a minor part whereas penicillins and cephalosporins are crucial agents for veterinary medicine of today [19].

2.1 Penicillin

There are several subgroups of penicillin. The four major subgroups are the benzylpenicillin, the phenoxypenicillin, the carboxypenicillin, and the acylamino-ureidopenicillin, with their respective derivates [5].

The first listed benzylpenicillin is acid-labile and therefore not for oral use at monogastric animals. Nevertheless, there is still an indication for the parenteral or local application against gram-positive bacteria such as *Staphylococcus* species. The acid-stable penicillin also known as oral penicillin, with a similar prescription as the benzylpenicillin subgroup. The carboxypenicillin, as the third listed subgroup, belongs to the so-called extended-spectrum penicillins. It is prescribed against gram-negative bacteria. However, its stability against beta-lactamases is very low [5]. The fourth subgroup, acylamino-ureidopenicillin, belongs as well to the extended-spectrum penicillins. It is not used in veterinary medicine [19]. It owns a high tissue penetration property that is a key advantage compared to all other subgroups. It is an antibiotic of last resort and used for special indications at hospitals [5].

2.2 Cephalosporins and cephamycins

The cephems represent a group of antibiotics that contains cephalosporin derivates as well as cephamycins. Since 1945 the cephalosporins were further developed into now five generations of antibiotics [20]. Striking formulas are amongst these: cephalexin, a first generation cephalosporin, is effective against penicillinase-producing staphylococci and streptococci, as well as against most Enterobacteriaceae [21]. The 2nd generation cephalosporin cefoxitin was discovered in 1972, and is as well applied for the treatment of methicillin-susceptible staphylococci and streptococci, but works for a broader variety of gram-negative bacteria such as *Haemophilus influenza*, *Enterobacter aerogenes* and some *Neisseria* species [22]. Two 3rd generation cephalosporins were tested in the present work. Ceftazidime and cefotaxime were discovered in the 1970s. These can be applied by injection and are used against joint infection, meningitis, pneumonia, sepsis and infection of the urinary tract. The spectrum includes also anaerobes such as *Bacteroides* species [23-24].

The 4th generation cephalosporins, such as cefepime, developed in the 1990s have an even greater activity than 3rd generation agents. It is applied against nosocomial infections, such

as pneumonia caused by multiple drug-resistant microorganisms [25]. The 5th generation cephalosporins were not investigated in the present study. However, substances as ceftolozane, were developed for intra-abdominal infections or pneumonia caused by resistant gram-negative bacteria [26].

2.3 Carbapenems

These highly effective antimicrobial agents were discovered from *Streptomyces cattleya* initally [27]. Although they exhibit a narrow spectrum against gram-positive bacteria, they can be applied against most Enterobacteriaceae and count to the drugs of last resort. They are applied against intra-abdominal infections, pneumonia and sepsis [28].

2.4 Beta-lactamase inhibitor (clavulanic acid, avibactam)

As described beta-lactamase is an enzyme responsible for bacterial resistance against beta-lactam antibiotics. It breaks open the ring structure of beta-lactam antibiotics, a strategy to impede the effect of beta-lactams. Pharmaceuticals were developed to inhibit the activity of beta-lactamases and are therefore called beta-lactamase inhibitors.

Clavulanic acid was described in 1974 and patented in 1981 [29]. It is a natural product in the metabolism of the bacterium *Streptomyces clavuligerus*. There its chemical structure acts as a suicide inhibitor binding to the active site of the beta-lactamase. This process restructures the clavulanic acid molecule, creating an even more reactive molecule, and finally inactivates the beta-lactamase permanently [29].

Avibactam is effective against Ambler class A beta-lactamases, e.g. cefotaximase-Munich (CTX-M), Temoneira beta-lactamase (TEM), sulphhydryl variable beta-lactamase (SHV), Ambler class C, aminopenicillin-inactivating cephalosporinase (AmpC), and selected Ambler class D beta-lactamases, e.g. oxacillin-hydrolyzing beta-lactamase (OXA-48). In contrary, it is not quite effective against Ambler class B metallo-beta-lactamases, e.g. Verona integron-encoded beta-lactamase (VIM) [30]. Only since 2013, it is used in combination with ceftazidime for the treatment of complicated urinary tract and severe intra-abdominal infections caused by antibiotic resistant pathogens [30].

An organism was identified as an ESBL-producer if there was a more or equal than threefold concentration-decrease in the minimal inhibitory concentration (MIC) for either antimicrobial

agent tested in combination with clavulanic acid versus the MIC of the agent when tested alone [14, 31]. As an example for a true ESBL-producer according to published guidelines by the American CLSI the listed a MIC of 8 mg/l ceftazidime and the more than three-fold concentration decrease in the MIC of 1 mg/l ceftazidime-clavulanic acid [15-16].

3 Measurement of bacterial resistance

The measuring of antimicrobial resistance *in vitro* is a complex laboratory method. The principal is to determine the degree of growth inhibition for a specific isolate in the presence of an antibiotic substance. This depends upon the tested bacterial species and initially requires a pure culture isolate. The mere MIC is then interpreted according to the bacterial species by breakpoints and classified into susceptible (S), intermediate (I) and resistant (R) [32]. If the available guidelines, e.g. published by the EUCAST do not provide breakpoints for a certain bacterial species, the pharmacokinetic and pharmacodynamic modeling is required. This is called a non-species-related breakpoint [17].

3.1 Agar Diffusion Method

The agar diffusion method is older than half a century and it is still in use today [33]. An antibiotic substance diffuses by gradient from a disk into the surrounding agar. The growth of a susceptible bacterial isolate will be inhibited at the zone of its individual MIC. The latter measured in millimeters is interpreted according to published guidelines into S-I-R status of each bacterial isolate [17]. Recent studies still use this method for phenotypic identification of ESBL- or AmpC-producing *E. coli* [34]. In the present study however, this method was not applied due to high sample volumes and comparability.

3.2 Epsilometer Test

The Epsilometer test, an *in vitro* diagnostic device, was developed and presented in 1988 for the first time. The elongated disk already includes the gradient of a specific antibiotic substance, which diffuses into the agar plate. Thus, an epsilon shaped inhibiting areola enables precise reading of the MIC directly on a scale at the disk. The reliability and reproducibility of the Epsilometer test is more precise compared to the original disk diffusion

method [35]. However, only a limited number, one or two, antibiotic substances are tested on one agar plate, making this method quite challenging for diversified routine testing. As well, the method takes more time and is also costlier than the disc diffusion method.

3.3 Broth Microdilution

The method of choice, as it was used in the present study, was the broth microdilution. Here, a standardized concentration of viable bacteria in a broth medium was transferred in each well of a microtiter plate. Each vial contained a specific concentration of different antibiotics (Fig. 4). The growth of the target isolate was therefore not restrained by any other parameter than its susceptibility. This method enabled the generation of MIC results at a high throughput rate and excellent reproducibility. Therefore, a broad variety of beta-lactam antibiotics and beta-lactamase inhibitors were compared on a large study panel [17, 36].

Figure 4: Setting of broth microdilution plate (Merlin) as used in the present study

	1	2	3	4	5	6	7	8	9	10	11	12
A	CEP 128	CMC 32/4	CAZ 128	CZC 32/4	CZB 32	CTX 128	C/C 32/4	CTB 32	MER 128	MEE 32	MEB 32	COX 32
B	CEP 64	CMC 16/4	CAZ 64	CZC 16/4	CZB 16	CTX 64	C/C 16/4	CTB 16	MER 64	MEE 16	MEB 16	COX 8
C	CEP 32	CMC 8/4	CAZ 32	CZC 8/4	CZB 8	CTX 32	C/C 8/4	CTB 8	MER 32	MEE 8	MEB 8	COX 4
D	CEP 16	CMC 4/4	CAZ 16	CZC 4/4	CZB 4	CTX 16	C/C 4/4	CTB 4	MER 16	MEE 4	MEB 4	ERT 1
E	CEP 8	CMC 2/4	CAZ 8	CZC 2/4	CZB 2	CTX 8	C/C 2/4	CTB 2	MER 8	MEE 2	MEB 2	ERT 0,5
F	CEP 4	CMC 1/4	CAZ 4	CZC 1/4	CZB 1	CTX 4	C/C 1/4	CTB 1	MER 4	MEE 1	MEB 1	GC/B
G	CEP 2	CMC 0,5/4	CAZ 2	CZC 0,5/4	CZB 0,5	CTX 2	C/C 0,5/4	CTB 0,5	MER 2	MEE 0,5	MEB 0,5	GC/E
H	CEP 1	CMC 0,25/4	CAZ 1	CZC 0,25/4	CZB 0,25	CTX 1	C/C 0,25/4	CTB 0,25	MER 1	MEE 0,25	MEB 0,25	GC

key: cefepime (CEP), cefepime + clavulanic acid (CMC), ceftazidime (CAZ), ceftazidime + clavulanic acid (CZC), ceftazidime + 3-APB (CZB), cefotaxime (CTX), cefotaxime + clavulanic acid (C/C), cefotaxime + 3-APB (CTB), meropenem (MER), meropenem + EDTA (MEE), meropenem + 3-APB (MEB), cefoxitin (COX), ertapenem (ERT), growth control (GC), concentrations in mg/l

4 Clinical aspects regarding the usage of antibiotics

Beta-lactam antibiotics are highly effective drugs regarding the treatment of bacterial infections in small animals. They are therefore used frequently in veterinary medicine of companion, farm or exotic animals [12, 19, 37]. The tested antibiotic substances such as cefoxitin (or oxacillin), cefotaxime, ceftazidime, cefepime, and their combinations with clavulanic acid are frequently applied to dogs when necessary for the treatment of an infection. Besides, it needs to be considered that there is a well-known cross-hypersensitivity potential of beta-lactams for these animals [19, 37].

However, the increasing antimicrobial resistance especially against 3rd generation cephalosporins, here cefotaxime, ceftazidime, and 4th generation cephalosporins, here cefepime, is currently thought as a serious problem, and application in general needs to be well-considered [19, 38]. Even more, due to close proximity of companion animals to humans, the treatment of disease-causing ESBL-producers in companion animals is indisputable [39-41]. And also, the risk of transfer of genetic elements carrying antimicrobial resistance from or to companion animals due to their close contact with humans, interspecies transmission, must not be underestimated [42].

5 Prevalence of ESBL-producing bacteria

For infectious microorganisms, we often find specific reservoirs such as the horse in glanders, ruminants in brucellosis or the hare in tularemia [1]. As antimicrobial resistance is not a host specific property, bacteria expressing this feature may be identified in a variety of reservoirs, such as humans, livestock and companion animals, birds, but as well food, feed or even water [43].

5.1 Birds and poultry

The prevalence of ESBL-producing bacteria has been published for wild birds as well as for agricultural birds, such as poultry [44]. Regarding results of a screening study in the Netherlands, all sampled broiler farms revealed a rate of 80% positive swabs and fecal samples [45]. Similar results were recovered from a longitudinal study in Germany with a prevalence for ESBL-producing bacteria of up to 76%. This study additionally revealed that the prevalence

of these bacteria increases during the fattening period (35 days) of the birds [46]. Retrospective characterization of isolates from wild birds revealed a multidrug-resistant ESBL-producing *Salmonella* Serovar Corvallis from a black kite (*Milvus migrans*) in Germany [47]. In another study, more than 500 cloacal swabs were screened for carbapenemase-producing Enterobacteriaceae in Australia. A total of 120 isolates originating from ten bacterial species were characterized and interpreted as large-scale transmission of antimicrobial resistant bacteria into wildlife [48]. Further studies from the countries of Mongolia and Saudi Arabia as well provided data regarding ESBL-producing bacteria in wild birds [49]. Wild birds do play an important role in the transmission of infectious microorganisms due to migration over large distances. Then they do function as reservoir but also as vectors [50].

5.2 Farm animals

In contrast to the results regarding an increase of ESBL-producing bacteria during the 35-day fattening process in poultry, the prevalence of ESBL-producing bacteria decreased significantly in veal calves within an investigation time of eight and ten weeks [51]. The countrywide survey of resistant *Escherichia coli* revealed a prevalence of 25% for broilers, 3% for pigs, and 4% for cattle determined for slaughtered animals [52]. These results suggested a higher prevalence of resistant bacteria the more packed the animals were kept. However, the transmission dynamics in farm animals is highly complex, and cannot be calculated using only few parameters [51]. For humans one considerable transmission risk is the presence of resistant bacteria in the food chain [53]. A second risk for a special group of humans (e.g. veterinarians, farmers) is present regarding the close contact to animals.

5.3 Companion animals, dogs

ESBL-expressing *Escherichia coli* isolated from dogs were first described in 1988, following treatment of the dogs with beta-lactam antibiotics [54]. Since then, the presence of multidrug-resistant bacteria has been described repeatedly for sick, but also entirely healthy companion animals, including dogs [55-58]. One longitudinal study occurring over eleven months identified a variety of ESBL-producing Enterobacteriaceae in healthy dogs with highly dynamic fecal shedding patterns, occurring either continuously or periodically [36, 59]. Single drug resistant *Escherichia coli* have been isolated from dogs directly after antibiotic treatment

already. In a direct experiment, involving animals including 24 dogs shedding of *Escherichia coli* resistant to beta-lactam antibiotics were recovered in 100% of the animals after seven-day treatment with amoxicillin [60].

The dog is amongst the earliest companion animals in the history of man. Therefore, it coevolved in a variety of functions parallel to the human societies. Ever since, dogs were used for guarding, working, company, and finally of course for pleasure. In all these functions this animal is a potential reservoir and vector of pathogens as well as antimicrobial resistant bacteria [61].

III. Publication

The publication entitled “Phenotypic characterization and whole genome analysis of extended-spectrum beta-lactamase-producing bacteria isolated from dogs in Germany“ was published in PLOS ONE, a peer reviewed journal [36].

PLOS ONE

RESEARCH ARTICLE

Phenotypic characterization and whole genome analysis of extended-spectrum beta-lactamase-producing bacteria isolated from dogs in Germany

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Abstract

Asymptomatic colonization with extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae has been described for humans, various mammal species, and birds. Here, antimicrobial resistant bacteria were recovered from dog feces originating in Germany, Kosovo, Afghanistan, Croatia, and Ukraine, with a subset of mostly *E. coli* isolates obtained from a longitudinal collection over twelve months. *In vitro* antimicrobial resistance testing revealed various patterns of resistance against single or all investigated beta-lactam antibiotics, with none of the 101 isolates resistant against two tested carbapenem antibiotics. Whole genome sequence analysis revealed bacteria species-specific patterns for 23 antimicrobial resistance coding DNA sequences (CDS) that were unapparent from the *in vitro* analysis alone. Phylogenetic analysis of single nucleotide polymorphisms (SNP) revealed clonal bacterial isolates originating from different dogs, suggesting transmission between dogs in the same community. However, individual resistant *E. coli* clones were not detected over a period longer than seven days. Multi locus sequence typing (MLST) of 85 *E. coli* isolates revealed 31 different sequence types (ST) with an accumulation of ST744 (n = 9), ST10 (n = 8), and ST648 (n = 6), although the world-wide hospital-associated CTX-M beta-lactamase producing ST131 was not detected. Neither the antimicrobial resistance CDSs patterns nor the phylogenetic analysis revealed an epidemiological correlation among the longitudinal isolates collected from a period longer than seven days. No genetic linkage could be associated with the geographic origin of isolates. In conclusion, healthy dogs frequently carry ESBL-producing bacteria, independent to prior treatment, which may be transmitted between individual dogs of the same community. Otherwise, these antimicrobial resistant bacteria share few commonalities, making their presence eerily unpredictable.

Introduction

Beta-lactams are among the most popular antibiotics, worldwide, for the treatment of bacterial infections [1]. Unfortunately, multidrug-resistant bacteria producing extended-spectrum beta-lactamases (ESBL) are also prevalent worldwide [2]. Descriptions of ESBL isolates originating from patients in intensive care units of European hospitals were first published in the mid-1980s [1]. Since then, ESBL-producing Enterobacteriaceae have been identified from a plethora of sources, including humans, animals, food, feed, and other environmental sources [3–7].

ESBL-producing *Escherichia coli* isolated from dogs were first described in 1988, following treatment of the dogs with beta-lactam antibiotics [8]. Since then, the presence of ESBL-producing bacteria has been described repeatedly for sick, but also completely healthy companion animals, including dogs [4,9–13]. One longitudinal study occurring over six months identified a variety of ESBL-producing Enterobacteriaceae in healthy dogs with highly dynamic fecal shedding patterns, occurring either continuously or periodically [14].

Comprehensive characterization of ESBL-producing Enterobacteriaceae is critical for understanding transmission routes and persistence in potential reservoirs, as well as their potential to transfer multidrug-resistant genetic coding elements and/or cause disease [15]. To date, a variety of methods, including biochemistry, phage typing, serotyping, bacteriocin typing, analytical isoelectric focusing, and pulsed-field gel electrophoresis have been used for characterization [1, 9, 16–17]. However, the discriminatory power of these methods has been incomplete and the reproducibility among different laboratories low, limiting insight into the epidemiology of these bacteria [1]. High throughput whole genome sequencing provides an opportunity to gather much more comprehensive data on antimicrobial resistance carrying genetic elements in various bacteria. And, when coupled with appropriate epidemiological data, should allow for greater insight into the population dynamics of ESBL-producing bacteria [17–19]. In this study, we combine *in vitro* diagnostics with whole genome analysis to investigate the genetic diversity and antimicrobial resistance profiles of ESBL-producing bacteria from dogs living in close proximity to humans and gain a greater understanding of this overlooked source of antimicrobial resistance.

Material and methods

Strain isolation

ESBL-producing bacteria were exclusively isolated from fresh canine feces. As the dogs were not at all touched for this purpose, the Institutional Animal Care and Use Committee (IACUC) was not involved. The authorization of the sample collection regarding animals within any North Atlantic Treaty Organization (NATO) theatre of operations was given by direct NATO order and was to be executed by the military veterinary authorities, here authors of the present study, that must review which diseases were prevalent in the area to which animals will be deployed [20]. The collection within Germany was carried out within the area of caserns or on private land in the presence of and in accordance to the commanding officer or the respective landlord.

Dog feces from a community of 17 German (GER) military dogs, and from three additional military dogs living in a different community, was sampled over a twelve-month period, from April 2015 to March 2016. Within this longitudinal subset, fecal samples of all dogs were screened daily within the first week of investigation, then weekly during the first month, then monthly for six months, and, finally, once at the end of twelve months. The sampled dogs had no history of treatment over the previous twelve months. Additional, sporadic samples were collected from military dogs from other locations, including Croatia (CRO) and Ukraine

(UKR), and also from stray dogs from military operation zones in Afghanistan (AFG) and Kosovo (KOS) (S1 Table). Samples were collected from dog feces directly after voiding, and were processed in the laboratory within a maximum of six hours. Initial screening of fecal samples was carried out by direct inoculation on a selective Brilliance ESBL AGAR (Oxoid, Wesel, Germany) containing an antibiotic-mix, using a 10 μl inoculation loop. Plates were incubated at 37˚C, and putative isolates were harvested based on their colony morphology after 24 h according to the manufacturer's instructions. All morphologically suspicious isolates were picked, with at least three morphologically indistinguishable isolates selected per plate, if available. Selected isolates were then sub-cultured on Columbia sheep blood agar (Oxoid, Wesel, Germany). The tentative species of each isolate was determined via mass spectrometry using a MALDI Biotyper system (Bruker, Bremen, Germany).

The isolates were named according to their geographic origin (GER, UKR, KOS, CRO, AFG), individual source (military dog—MD, stray dog–SD, stray fox–SF, companion dog–CD, environmental–EN, and number indicating specific animal), year and month of isolation, bacterial species and a running number within the present project; e.g. GER_MD06_1505_Eco_007 (S1 Table).

Whole genome analysis confirmed the species identification for the investigated isolates except for two isolates: the *Enterobacter kobei* isolate GER_MD16_1505_Esp_090 was labeled *Enterobacter* sp. and the *Pseudomonas fulva* isolate AFG_SD02_1510_Psp_092 was labeled *Pseudomonas* sp. due to low measures of identity to reference genomes for each species.

In vitro antimicrobial susceptibility testing

All recovered isolates were tested *in vitro* for their antimicrobial resistance profile using the commercially available standard micro-dilution system, MICRONAUT-S Beta-Lactamases (Merlin, Berlin, Germany). This method included tests for six different singular antimicrobial substances, including, cefoxitin (COX), cefotaxime (CTX), ceftazidime (CAZ), cefepime (CEP), ertapenem (ERT), and meropenem (MER), and three additional combinations comprised of CTX, CAZ, and CEP tested in combination with clavulanic acid. The minimum inhibitory concentration (MIC) was determined for each isolate in accordance with the manufacturer's directions (Merlin, Berlin, Germany).

To assess the presence of multiple beta-lactamases (multiple resistance determinants) MIC values were interpreted according to the breakpoint-value standards set for drug selection and interpretation by the Clinical and Laboratory Standards Institute (CLSI, Wayne, PA, USA). Specifically, the CLSI *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, 27th Informational Supplement (M100-S27), and the VET01/ VET01-S2 guidelines, *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals*, presented by the Subcommittee on Veterinary Antimicrobial Susceptibility Testing were used [21–22]. Complete breakpoint values were solely available for *E.* coli and P. mirabilis and partially available for CEP and CAZ for *Pseudomonas aeruginosa.* However, no breakpoints were available for *Enterobacter* spp., *Aeromonas* sp. and *Pseudomonas* sp. other than *P. aeruginosa* [21]. By definition, extended-spectrum beta-lactamase is produced by a bacterium if more than a three twofold concentration decrease in a MIC is observed for either antimicrobial agent tested in combination with clavulanic acid versus the MIC of the agent when tested alone [22].

Genome sequencing, assembly, MLST

Whole genome sequencing was attempted for all isolates. DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), and sequenced on the Illumina MiSeq

and NextSeq platforms (TGen, Flagstaff, AZ, USA). Raw sequence data were assembled with a pipeline that includes Trimmomatic for read trimming [23], SPAdes v3.10.1 for contig assembly [24], Pilon for assembly polishing [25], and BLAST to identify potential sequence contamination [26]. *In silico* multi-locus sequencing typing (MLST) of identified *E. coli* genomes was carried out using a custom script (https://gist.github.com/jasonsahl/2eedc0ea93f90097890879e56b0c3fa3) that utilizes BLAST and the PubMLST database (https://pubmlst.org/) for *Escherichia coli* [26–27].

Screening for CDS associated with antimicrobial resistance and virulence factors

All recovered genome assemblies were screened for antibiotic resistance genes with ABRicate (https://github.com/tseemann/abricate), using the ResFinder database (downloaded 2017 July 8) [28]. In addition, virulence gene profiles were determined by screening the genome assemblies for selected virulence coding DNA sequences (CDSs) with the large-scale blast score ratio (LS-BSR) pipeline [29] using the BLAT alignment option [30]. Screened virulence genes included 35 publicly available CDSs for fimbriae, toxins, and other proteins responsible for adhesion, agglutination, gene transfer, or iron acquisition. A CDS was considered as present within a genome if the blast score ratio was above 0.8 [31].

Phylogenetic analyses

Phylogenetic analyses were applied to all of the recovered *E. coli* sequences to identify genetic relationships among the isolates. The *E. coli* genomes were compared to a reference, K-12 W3110 (GCA_000010245.1), and core genome single nucleotide polymorphisms (SNPs) were identified [32]. Specifically, sequencing reads were aligned to the reference with BWA-MEM [33]. SNPs were called using the UnifiedGenotyper method in GATK [34–35]. Putative SNP positions with less than 10X coverage or allele proportions less than 90% were filtered from the analysis. Any SNP identified from duplicated regions of the reference, as identified through NUCmer [36] self-alignments, were filtered from downstream analyses. All of the SNP detection methods were performed in conjunction with the NASP pipeline [37]. Phylogenies were inferred from the identified SNPs with IQ-TREE v 1.4.4 using the identified best-fit model, TVM+ASC+G4 (S3 Table) [38].

Results

Isolates

In total, 101 bacterial isolates were recovered using the selective Brilliance ESBL AGAR between January 2015 and June 2016 (S1 Table). Of these, 75 originated from 16 German military dogs, with an additional five originating from two companion dogs (GER_CD71, GER_CD72) living in the same household as German military dog GER_MD77. Of the foreign isolates, six originated from stray dogs (n = 2), shelter dogs (n = 3), and a stray fox (n = 1) in Kosovo; eight originated from stray dogs in Afghanistan; three originated from a Ukrainian military dog located in Kosovo; and two originated from a Croatian military dog located in Afghanistan (S1 Table). Two additional isolates originated from routine hygiene samples in Germany, and were considered as outgroups of non-animal origin.

Isolates were recovered from 16 of the 20 tested German military dogs. However, repeat isolation of ESBL-producing bacteria from samples taken on different dates was only successful for five of the 17 German military dogs in the longitudinal study (29%) (S1 Table). The longitudinal collection identified twelve isolates from GER_MD01 over a period of eleven months,

five isolates from GER_MD02 over a period of seven months, ten isolates from GER_MD03 over a period of three months, four isolates from GER_MD06 over a period of seven months, and eight isolates from GER_MD14 over a period of seven months (S1 Table).

A total of 31 isolates were collected from either the same dog or household within a single month of sampling, allowing for an examination of ESBL-diversity within a single dog and/or household over a short period of time. These included three isolates from GER_MD07, three isolates from GER_MD08, 13 isolates from GER_MD11, two isolates from GER_MD17, seven isolates from GER_MD77 or his companions GER_CD71 and GER_CD72, and three isolates from UKR_MD01 (S1 Table).

Identification of the 101 isolates revealed 93 *Escherichia coli*, one *Proteus mirabilis*, two *Enterobacter cloacae*, one *Enterobacter* sp. (all family Enterobacteriaceae), one *Aeromonas caviae*, one *Aeromonas hydrophila*, one *Pseudomonas aeruginosa*, and one *Pseudomonas* sp. (S1 Table). For two isolates, the identification was possible only on genus level due to contradictory results based on the MALDI-TOF and the molecular approach.

Antimicrobial susceptibility

MICs for the entire antibiotic test panel were recovered for all isolates (S2 Table). Interpretation of MICs was carried out for the 94 isolates (*E. coli*, *P. mirabilis*) in accordance with CLSI criteria. Due to limited or missing MIC values in the CLSI guidelines, interpretation was restricted for CAZ and CEP for the *P. aeruginosa* isolate and could not be performed for the *Enterobacter*, *Aeromonas* and *Pseudomonas* non-*aeruginosa* isolates (S2 Table). For COX, representing cephamycin antibiotics within the 2nd generation of cephalosporins, 13 isolates (14%) were resistant and 81 (86%) were susceptible (S2 Table). For CTX, representing 3rd generation cephalosporins, 91 isolates (97%) were resistant, one (1%) was susceptible, and two (2%) had an intermediate state. For CAZ, 34 isolates (36%) were resistant, 51 (54%) were susceptible, and ten had an intermediate state (S2 Table). For CEP, a 4th generation cephalosporin, 87 isolates (92%) were resistant, six (6%) were susceptible, and two (2%) had an intermediate state (S2 Table). For the carbapenems, ERT and MER, all tested isolates were susceptible (S2 Table). Tests of CTX, CAZ, and CEP with the addition of 4 μg/ml clavulanic acid to inhibit beta-lactamase activity, revealed 88 (93%) out of 95 isolates to be real ESBL-producers in the *in vitro* system and according to the CLSI guidelines (S2 Table) [21–22]. One isolate, GER_MD01_1509_Eco_059, was susceptible to all of the tested substances (S2 Table).

Genome assembly and CDS identification

Draft genome assemblies were generated for 93 isolates, with the remaining eight isolates excluded due to poor sequence quality (S3 Table). Of these, 85 were identified as *E. coli* genomes. Genome assemblies were submitted to GenBank and raw data was submitted to the sequence read archive (see S3 Table for individual accession numbers).

Use of ABRicate and the ResFinder database revealed sequence hits for 23 of 1,309 screened CDSs for beta-lactamases amongst the genome assemblies (Table 1 and S4 Table). Regarding class A beta-lactamase genes, 74 of 85 analyzed *E. coli* genomes possessed at least one CTX-M-type beta-lactamase CDS, with 33 positive for $bla_{\text{CTX-M-1}}$, 28 positive for $bla_{\text{CTX-M-15}}$, eleven positive for $bla_{\text{CTX-M-14}}$, two positive for $bla_{\text{CTX-M-3}}$, and one positive for $bla_{\text{CTX-M-2}}$. One isolate was positive for $bla_{\text{SFO-1}}$ and another for $bla_{\text{SHV-12}}$. Forty isolates possessed $bla_{\text{TEM-1}}$-type beta-lactamase CDSs, with two positive for $bla_{\text{TEM-1A}}$ and 38 positive for $bla_{\text{TEM-1B}}$ (Table 1 and S4 Table). Regarding class B beta-lactamases, one *A. hydrophilia* genome was positive for $bla_{\text{Cph-A1}}$ and three *E. coli* genomes were positive for $bla_{\text{VIM-1}}$ (Table 1). Ten genomes were positive for class C beta-lactamase CDSs, including three *E. coli* genomes positive for $bla_{\text{ACC-1}}$

Table 1. Prevalence of 23 specific beta-lactamase (BL) genes coding for antimicrobial resistance showing a clear species specificity (also S4 Table).

category	BL, extended-spectrum BL (ESBL)	specific resistance gene	positive strains (n) out of 93	bacterial species
class A BL (penicillinase)	cefotaximase-Munich, ESBL	$bla_{\text{CTX-M-1}}$	33	*Escherichia coli*
		$bla_{\text{CTX-M-2}}$	1	*Escherichia coli*
		$bla_{\text{CTX-M-3}}$	2	*Escherichia coli*
		$bla_{\text{CTX-M-14}}$	11	*Escherichia coli*
		$bla_{\text{CTX-M-15}}$	28	*Escherichia coli*
	Serratia fonticola class A BL	$bla_{\text{SFO-1}}$	1	*Aeromonas hydrophila*
	sulphydryl variable class A BL	$bla_{\text{SHV-12}}$	1	*Escherichia coli*
	Temoneira, class A BL	$bla_{\text{TEM-1A}}$	2	*Escherichia coli*
		$bla_{\text{TEM-1B}}$	38	*Escherichia coli*
class B BL (carbapenemase)	carbapenem-hydrolyzing metallo BL	$bla_{\text{Cph-A1}}$	1	*Aeromonas hydrophila*
zinc dependent	Verona integron-encoded metallo BL	$bla_{\text{VIM-1}}$	3	*Escherichia coli*
class C BL (cephalosporinase)	Ambler class C-1 cephalosporin-hydrolyzing class C BL	$bla_{\text{Acc-1}}$	3	*Escherichia coli*
	ampicillin type cephalosporin-hydrolyzing class C BL	$bla_{\text{Act-7}}$	1	*Enterobacter cloacae*
		$bla_{\text{Act-14}}$	1	*Enterobacter cloacae*
	aminopenicillin-inactivating (Amp) cephalosporinase	bla_{AmpH}	1	*Aeromonas hydrophila*
	cephamycinase, plasmid derived pYMG-1 *bla*	$bla_{\text{CMY-2}}$	1	*Escherichia coli*
	methoxy-/ imino-Res; cephalosporin-hydrolyzing class C BL	$bla_{\text{MIR-6}}$	1	*Enterobacter* sp.
	moxalactam-inactivating cephalosporinase	$bla_{\text{MOX-5}}$	1	*Aeromonas hydrophila*
		$bla_{\text{MOX-6}}$	1	*Aeromonas caviae*
	Pseudomonas aeruginosa cephalosporinase	bla_{PAO}	1	*Pseudomonas aeruginosa*
class D BL	oxacillin-hydrolyzing BLs	$bla_{\text{OXA-1}}$	18	*Escherichia coli*
		$bla_{\text{OXA-50}}$	1	*Pseudomonas aeruginosa*
		$bla_{\text{OXA-504}}$	1	*Aeromonas caviae*

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and single isolates positive for $bla_{\text{ACT-7}}$, $bla_{\text{ACT-14}}$, $bla_{\text{CMY-2}}$, $bla_{\text{MIR-6}}$, $bla_{\text{MOX-5}}$, $bla_{\text{MOX-6}}$, bla_{PAO}, and *ampH*, respectively (Table 1 and S4 Table). Twenty isolates were positive for class D beta-lactamase CDSs, including 18 *E. coli* genomes positive for $bla_{\text{OXA-1}}$, one *P. aeruginosa* positive for $bla_{\text{OXA-50}}$, and one *A. caviae* positive for $bla_{\text{OXA-504}}$ (Table 1 and S4 Table). Only two isolates, the GER_MD10_1505_Pmi_049, and the GER_EN02_1501_Eco_088 were negative for all of the 1,309 screened beta-lactamase CDSs (Table 1 and S4 Table).

Patterns in the identified antimicrobial resistance CDSs suggested bacterial species specificity (Table 1). The two *E. cloacae* isolates were the only isolates to possess $bla_{\text{ACT-7}}$ and $bla_{\text{ACT-14}}$, respectively. Likewise, the further *Enterobacter* sp. isolate was the only isolate to possess $bla_{\text{MIR-6}}$, the *P. aeruginosa* isolate was the only isolate to possess bla_{PAO} and $bla_{\text{OXA-50}}$, and the *A. caviae* isolate was the only isolate positive for $bla_{\text{MOX-6}}$ and $bla_{\text{OXA-504}}$. Similarly, the *A. hydrophila* isolate was the only isolate positive for $bla_{\text{SFO-1}}$, $bla_{\text{Cph-A1}}$, $bla_{\text{MOX-5}}$, and *ampH*, four antimicrobial resistance coding beta-lactamase genes and the highest number detected in a single isolate (Table 1).

Sequence hits for seven of 35 screened virulence CDSs were detected among the genome assemblies originating from the present study group (Table 2). Sequence hits included eleven isolates positive for an adhesion protein CDS (i.e., the long polar fimbriae *lpfA*), one isolate positive for the agglutination protein temperature sensitive hemagglutinin CDS (*tsh*). A CDS

Table 2. Virulence genes detected in the isolates of the present study using whole genome sequence analysis.

Protein function	Gene	Full name and effect	Number of isolates carrying the respective gene	Accession Number
adhesion	lpfA	long polar fimbriae	11	AB161111.1
agglutination	tsh	temperature sensitive hemagglutinin, autotransporter protein	1	AF218073.1
gene transfer	argW	tRNA gene; site specific integration into chromosome and horizontal gene transfer	20	U11296.1
iron acquisition	ireA	Siderophore, iron carrier receptor	4	KU295572.1
toxin	estA	*E. coli* heat stable toxin A/ +variant	1 (environment)	AF005091.1
toxin	STp	ETEC heat-stable enterotoxin	1 (environment)	FN649417.1:c57269-57051
toxin	senB	*Shigella* enterotoxin B	4	Z54195.1

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catalyzing site specific integration into chromosome and responsible for horizontal gene transfer (*argW* tRNA gene) was detected in 20 of the isolates [39–40]. The CDS for iron acquisition, and iron carrier system, siderophore receptor A (*ireA*) was detected in four of the isolates [41]. Regarding toxin production, one isolate was positive for *E. coli* heat-stable (*ST*) enterotoxin A (*estA*) and the ETEC heat-stable enterotoxin (*STp*). Finally, four isolates were positive for *Shigella* enterotoxin B (*senB*) (Table 2). No isolate possessed more than two of the detected virulence CDSs (S1 Table).

MLST, SNPs and phylogenetic analysis

The MLST, SNP and phylogenetic analyses were limited to the *E. coli* sequences. Of the 85 identified *E. coli*genomes, 81 could be classified as one of 31 out of > 7,000 known *E. coli* sequence types (STs) based on MLST. The most frequently identified STs were ST744 (n = 9), ST10 (n = 8), ST648 (n = 6), ST58 (n = 4), and ST315 (n = 4), with the remaining 26 STs represented ≤3 times among the 85 genomes (Fig 1 and S1 Table). The remaining four *E. coli* genomes each contained one or two novel MLST alleles, resulting in three new, as yet unassigned STs (S1 Table). Phylogenetic analysis of 215,629 concatenated SNPs identified among the core genome of the analyzed *E. coli* isolates revealed clustering consistent with the identified STs (Fig 1). Within MLST ST10, ST101, and ST58, the SNP analysis revealed higher discriminatory power than pure MLST. Isolates belonging to these STs were collected on different dates, from different dogs and possessed different resistance CDSs (Fig 1, S3 Table). No clustering according to the geographic origin was observed among the study isolates, as most isolates from KOS, AFG, UKR, and CRO revealed different MLST STs, CDSs contents, and phylogenetic SNP clustering (Fig 1 and S1 Table).

In three cases, identical clones were detected from different dogs living in close contact. First, the combination of $bla_{\text{CTX-M-14}}$ and $bla_{\text{TEM-1B}}$ was found in nine ST744 clonal isolates originating from five different dogs isolated within the same month (Fig 1 and S1 Table). Second, the six ST648 isolates, originating from two different dogs in the same month, were the only isolates found to contain the combination of $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ (Fig 1 and S1 Table). Finally, three ST410 isolates, collected from three different dogs over five months, were all found to contain $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$. However, the isolate recovered five months after the other two did differ somewhat in that it was found to lack the *senB* gene (Fig 1 and Table 2). In one household, three dogs shed three ESBL-producing *E. coli* with identical MLST ST58 within 18 days, but these possessed SNP and gene content differences (GER_MD77_1507_Eco_075, GER_CD72_1507_082) (Fig 1 and S1 and S3 Tables). Focusing only on clonal isolates within longitudinal reshedding in individual dogs, a maximum isolation-time difference of seven days could

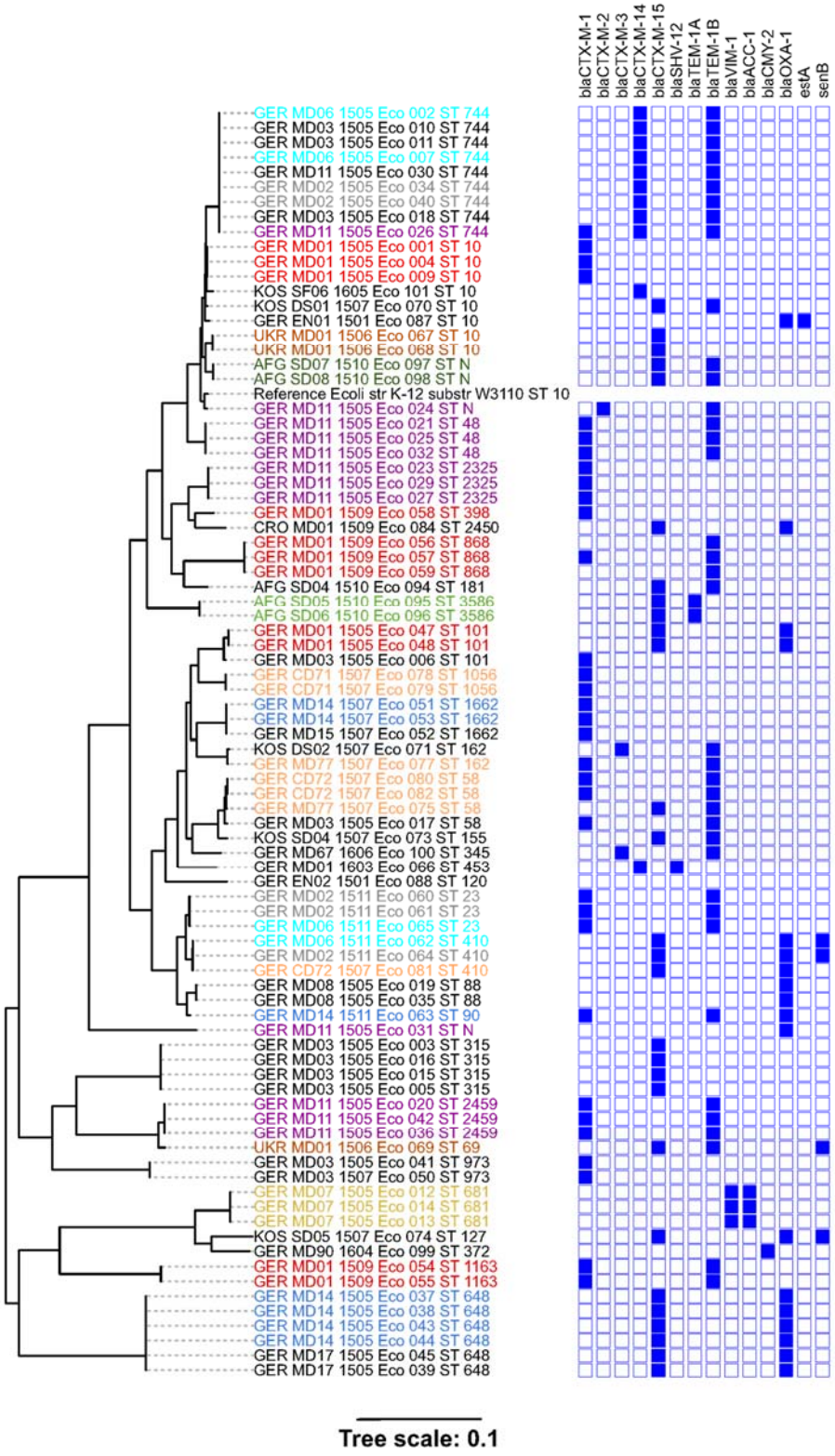
blaCTX-M-1
blaCTX-M-2
blaCTX-M-3
blaCTX-M-14
blaCTX-M-15
blaSHV-12
blaTEM-1A
blaTEM-1B
blaVIM-1
blaACC-1
blaCMY-2
blaOXA-1
estA
senB
GER MD06 1505 Eco 002 ST 744
GER MD03 1505 Eco 010 ST 744
GER MD03 1505 Eco 011 ST 744
GER MD06 1505 Eco 007 ST 744
GER MD11 1505 Eco 030 ST 744
GER MD02 1505 Eco 034 ST 744
GER MD02 1505 Eco 040 ST 744
GER MD03 1505 Eco 018 ST 744
GER MD11 1505 Eco 026 ST 744
GER MD01 1505 Eco 001 ST 10
GER MD01 1505 Eco 004 ST 10
GER MD01 1505 Eco 009 ST 10
KOS SF06 1605 Eco 101 ST 10
KOS DS01 1507 Eco 070 ST 10
GER EN01 1501 Eco 087 ST 10
UKR MD01 1506 Eco 067 ST 10
UKR MD01 1506 Eco 068 ST 10
AFG SD07 1510 Eco 097 ST N
AFG SD08 1510 Eco 098 ST N
Reference Ecoli str K-12 substr W3110 ST 10
GER MD11 1505 Eco 024 ST N
GER MD11 1505 Eco 021 ST 48
GER MD11 1505 Eco 025 ST 48
GER MD11 1505 Eco 032 ST 48
GER MD11 1505 Eco 023 ST 2325
GER MD11 1505 Eco 029 ST 2325
GER MD11 1505 Eco 027 ST 2325
GER MD01 1509 Eco 058 ST 398
CRO MD01 1509 Eco 084 ST 2450
GER MD01 1509 Eco 056 ST 868
GER MD01 1509 Eco 057 ST 868
GER MD01 1509 Eco 059 ST 868
AFG SD04 1510 Eco 094 ST 181
AFG SD05 1510 Eco 095 ST 3586
AFG SD06 1510 Eco 096 ST 3586
GER MD01 1505 Eco 047 ST 101
GER MD01 1505 Eco 048 ST 101
GER MD03 1505 Eco 006 ST 101
GER CD71 1507 Eco 078 ST 1056
GER CD71 1507 Eco 079 ST 1056
GER MD14 1507 Eco 051 ST 1662
GER MD14 1507 Eco 053 ST 1662
GER MD15 1507 Eco 052 ST 1662
KOS DS02 1507 Eco 071 ST 162
GER MD77 1507 Eco 077 ST 162
GER CD72 1507 Eco 080 ST 58
GER CD72 1507 Eco 082 ST 58
GER MD77 1507 Eco 075 ST 58
GER MD03 1505 Eco 017 ST 58
KOS SD04 1507 Eco 073 ST 155
GER MD67 1606 Eco 100 ST 345
GER MD01 1603 Eco 066 ST 453
GER EN02 1501 Eco 088 ST 120
GER MD02 1511 Eco 060 ST 23
GER MD02 1511 Eco 061 ST 23
GER MD06 1511 Eco 065 ST 23
GER MD06 1511 Eco 062 ST 410
GER MD02 1511 Eco 064 ST 410
GER CD72 1507 Eco 081 ST 410
GER MD08 1505 Eco 019 ST 88
GER MD08 1505 Eco 035 ST 88
GER MD14 1511 Eco 063 ST 90
GER MD11 1505 Eco 031 ST N
GER MD03 1505 Eco 003 ST 315
GER MD03 1505 Eco 016 ST 315
GER MD03 1505 Eco 015 ST 315
GER MD03 1505 Eco 005 ST 315
GER MD11 1505 Eco 020 ST 2459
GER MD11 1505 Eco 042 ST 2459
GER MD11 1505 Eco 036 ST 2459
UKR MD01 1506 Eco 069 ST 69
GER MD03 1505 Eco 041 ST 973
GER MD03 1507 Eco 050 ST 973
GER MD07 1505 Eco 012 ST 681
GER MD07 1505 Eco 014 ST 681
GER MD07 1505 Eco 013 ST 681
KOS SD05 1507 Eco 074 ST 127
GER MD90 1604 Eco 099 ST 372
GER MD01 1509 Eco 054 ST 1163
GER MD01 1509 Eco 055 ST 1163
GER MD14 1505 Eco 037 ST 648
GER MD14 1505 Eco 038 ST 648
GER MD14 1505 Eco 043 ST 648
GER MD14 1505 Eco 044 ST 648
GER MD17 1505 Eco 045 ST 648
GER MD17 1505 Eco 039 ST 648
Tree scale: 0.1

Fig 1. Phylogenetic tree of 85 ESBL-producing *E. coli*. Phylogenetic analysis based on 215,629 concatenated SNPs revealed clustering according to their MLST sequence type. *E. coli* K-12 substr W3110 was used as reference strain. All genome sequences except for the reference strain, and the environmental isolate GER_EN02_1501 revealed genes coding for antimicrobial resistance.

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be shown for five dogs (GER_MD03, GER_MD07, GER_MD08, GER_MD11, GER_MD14) (Fig 1 and S1 Table).

Discussion

We collected 101 bacterial isolates during a 12-month ESBL-screening study of clinically healthy dogs and characterized their antimicrobial resistance phenotypes and genotypes through *in vitro* testing and whole genome sequence analysis. Here, 16 of 20 German military dogs (80%) that had no history of medical treatment for the previous twelve months were found to shed ESBL-producing bacteria at least once within the study period (S1 Table). Although a high prevalence of ESBL-producing bacteria is suspected in livestock, our findings were surprising considering that the investigated animals were clinically healthy and untreated [42]. Also concerning was the result that 9% of the characterized *E. coli* isolates from clinically healthy German dogs were completely resistant against all tested cephalosporins (COX, CTX, CAZ, and CEP) (S2 Table). As the microbiological resistance against third-generation cephalosporins in European countries was stated as generally low in a review of 2012 data provided by the European Centers for Disease Control (ECDC), this result can be interpreted as a trend towards increasing multidrug resistance [43].

The overall trends of increasing antimicrobial resistance have led to several actions in recent years. In 2015, increasing concern on the animal welfare consequences of antimicrobial resistance in bacteria from animal sources led to the establishment of a sub-committee for Veterinary Antimicrobial Susceptibility Testing (VetCAST) of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [44]. In 2018, the national German veterinary pharmacy regulation law was enforced. According to this law, if a veterinarian applies antibiotics to animals, the MIC of bacterial isolates must be determined in case of repeated or change of medication, rededication, or regarding therapy of flocks or regarding animals bred for specific purposes [45]. This enforcement was aimed at a reduction of the use of antibiotics, but as well as at avoiding an increase of antimicrobial resistance through non-suitable therapy. Since 2014, the amounts and application of antibiotics in animal husbandry in Germany are officially collected in a large database. The Federal Veterinary Surgeons' Association regularly publish guidelines for the prudent use of veterinary antimicrobial drugs, and may consider data regarding the use, but also antimicrobial resistance [46]. The data of the present study contribute to comprehend trends within the complex field of antimicrobial resistance.

Resistance against single antibiotics within the class of cephalosporins was common among the investigated isolates, with 14% of the evaluated isolates resistant to COX and 92% resistant to CEP (S2 Table). COX is a 2nd generation cephamycin, frequently used in the treatment of dogs and other companion animals [46]. CEP is a 4th generation cephalosporin limited to use in humans, making the 92% resistance rate observed here unexpectedly high (S2 Table) [47]. These results should be considered when revising the drug application recommendations for human and animal patients [21].

Among the currently available beta-lactams, the carbapenems, such as ERT and MER, are antibiotics of last resort. They are unique in that they are resistant to a high degree against hydrolysis by most beta-lactamases. They can sometimes act as "slow substrates" or inhibitors of beta-lactamases, and, yet, still target penicillin-binding proteins [2]. Although carbapenems

are limited to use in humans only, off-label use or prescription may allow animals to be treated with these antibiotics [48]. In this study, we did not find any carbapenem resistance using the *in vitro* microbouillon dilution method (S2 Table). The *in silico* analysis detected similarly low levels of resistance, identifying only four isolates with a single carbapenemase CDS each (S4 Table). This suggests that dogs do not represent a likely source for the high rates of carbapenem resistance that have been published for hospital-acquired strains [49].

Antimicrobial resistance–*in vitro* and *in silico* analyses

Initial isolate selection was based on growth on supposedly ESBL- selective Brilliance ESBL AGAR (Oxoid, Wesel, Germany) containing an unknown antibiotic-mix. The *in vitro* analysis and subsequent interpretation according to current CLSI guidelines revealed 88 out of 95 isolates to be actual ESBL-producers [21–22]. As there were no interpretation guidelines available for six of the investigated bacterial isolates, including the *Enterobacter* spp., the *Aeromonas spp*., and *Pseudomonas* species other than *P. aeruginosa* isolates, we did not assign these as ESBL-producers in S2 Table [21–22]. However, for one out of these isolates (*Aeromonas hydrophila)*, ESBL-activity according to the rule "more than a three twofold concentration decrease comparing growth in the presence of CTX and CTX in combination with clavulanic acid" was observed (S2 Table) [21]. Pure antimicrobial resistance without ESBL-activity revealed five out of the investigated isolates. One more isolate, GER_EN01_1501_Eco_087, revealed an intermediate status, and another isolate, GER_MD01_1509_Eco_059, did not even reveal antimicrobial resistance in the *in vitro* testing. Finally, the *Pseudomonas aeruginosa* isolate, GER_MD14_1510_Pae_083, did not reveal ESBL-activity, it was considered as a susceptible isolate according to the CLSI guidelines for CAZ and CEP (S2 Table) [21]. These results indicate some lack of specificity for the selective Brilliance ESBL AGAR (Oxoid, Wesel, Germany). We compared the *in vitro* results with the detection of ESBL-specific CDSs in the *in silico* analysis (S5 Table). The susceptible isolate GER_MD01_1509_Eco_059, and the intermediate isolate GER_EN01_1501_Eco_087 revealed a single ESBL-CDS each, $bla_{\text{TEM-1B}}$ and $bla_{\text{OXA-1}}$, respectively (S4 Table). In contrast, the two *in vitro* antimicrobial resistant isolates GER_EN02_1501_Eco_088 and GER_MD10_1505_Pmi_049 did not reveal any ESBL-CDSs at all (S5 Table). Although the results from the two methodologies do not match entirely, we consider this a fairly high level of concordance between the *in vitro* and *in silico* analyses. It further suggests that the initial screening method for ESBL-producers was not highly specific, as eight isolates could grow on the selective media, but did not reveal true ESBL-properties (S2 Table).

The isolates GER_MD03_1507_Eco_050 and GER_MD11_1505_Eco_023 were found to possess only a single beta-lactamase gene (S4 Table). However, they revealed multiple resistance *in vitro*, against CTX and CEP, and were identified as ESBL-producers (S2 Table). *In vitro* and *in silico* correlation is therefore still too complex to predict a particular resistance from the result of a single detected beta-lactamase gene. Nevertheless, amongst the *E. coli* ESBL-producers, the most frequent ESBL-specific CDSs were $bla_{\text{CTX-M1}}$, $bla_{\text{CTX-M15}}$, $bla_{\text{TEM-1B}}$, and $bla_{\text{OXA-1}}$ in the present study, as it has been published (Table 1) [3, 11].

The investigated isolates belonged to six different bacterial species. Noticeably, the *in silico* results showed strict bacterial species-specific CDS patterns regarding antimicrobial resistance (Table 1). Although species specificity has been described for some of these resistance genes such as the "*Pseudomonas aeruginosa* cephalosporinase" (bla_{PAO}) [50], other classes can be found within various bacterial species belonging to the family Enterobacteriaceae such as the "sulphydryl variable class A beta-lactamase" (bla_{SHV}) [1]. Finally, some beta-lactamases, such as the "oxacillin-hydrolyzing beta-lactamase" (bla_{OXA}), and the "cefotaximase-Munich extended-spectrum beta-lactamase" ($bla_{\text{CTX-M}}$), were described for genetically distant bacterial

genera such as the Gram-positive *Enterococcus* and Gram-negative *Escherichia* [51]. Apart from their presence, enzymes may also vary regarding their kinetic activity. The "Temoneira class A beta-lactamases" (TEM-1) are able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and have negligible activity against extended-spectrum cephalosporins. Similar findings have been published for the CTX-M and OXA beta-lactamase subtypes [1]. The isolate GER_MD90_1604_Eco_099 was revealed to be resistant against 2nd and 3rd generation cephalosporins, COX, CTX, and CAZ (S2 Table). As previously published, a single resistance CDS, the cephamycinase bla_{CMY-2}, was likely responsible for this phenotype. Interestingly, this isolate was not an ESBL-producer by definition (S4 Table) [14]. Therefore, neither of the methods, either the phenotypic characterization nor whole genome analysis, can completely replace the other due to lack of crucial information. Thus, it is currently not possible to predict the phenotype using pure whole genome analysis and vice versa. However, due to its relevance for clinical diagnostics and treatment recommendations, the *in vitro* analysis will likely remain the gold standard at this time [21]. Drawbacks to this method include the fact that inoculum effects and *in vitro* conditions may affect MIC measurements, which may obscure a true underlying resistance genotype in various bacterial species [51–54]. In addition, non-Enterobacteriaceae organisms are currently not considered in CLSI guidelines for ESBL detection, impeding treatment recommendations for clinical patients affected by other species [1, 21].

In previous studies, PCR detection was used to identify individual CDSs of beta-lactamase subgroups of bla_{CTX-M}, bla_{CMY}, bla_{TEM}, bla_{SHV}, bla_{PSE}, bla_{OXA}, bla_{AmpC}, bla_{ACC} in isolates originating from companion animals [3, 4, 10–12, 51, 55–60]. By utilizing whole genome sequencing, we were able to identify seven additional beta-lactamase types and subtypes, including, bla_{SFO}, bla_{Cph}, bla_{VIM}, bla_{Act}, bla_{MIR}, bla_{MOX}, and bla_{PAO} (Table 1 and S4 Table). Similar findings have been published after the analysis of human derived ESBL-producing bacteria, reflecting the superior detection capabilities offered by whole genome sequence analysis [17]. In summary, not using whole genome sequence analysis, an investigator risks missing crucial information concerning antibiotic resistance that could be helpful and sometimes even crucial for subsequent epidemiological interpretation.

Prevalence of specific MLST STs and resistance genes

By the year 2000, a CTX-M beta-lactamase producing ST131 *E. coli* was recognized as a clone with worldwide prevalence, with about half of all hospital acquired ESBL-infections associated with this sequence type [17, 61–63]. In the present study, ST131 was not detected among the dog-derived isolates, suggesting that this ST might be less adapted to the canine host [64]. The two most common MLST ST identified in the present study were ST744 and ST10, with nine and eight isolates among the 85 isolates, respectively (Fig 1 and S1 Table). Several predominant ESBL-producing *E. coli* lineages have been identified for animals. The MLST ST10 was repeatedly isolated from pigs in Ireland, ST410 from small animals in Switzerland, and finally ST38 and ST131 from poultry and small animals in the Netherlands [51, 65–67]. As the MLST ST10 dog-isolates of the present study originated from Germany, Kosovo, Ukraine and Afghanistan, a significant geographic cumulation of MLST ST10 cannot be concluded from the present data (Fig 1 and S1 Table).

Regarding the transmission and prevalence of certain beta-lactamase subtypes, it has been suggested that human isolates hosting the CTX-M beta-lactamase subtypes vary by geographic origin [17]. In Germany, the plasmid coded $bla_{CTX-M-15}$ gene is the most frequent subtype originating from human patient isolates [7, 62]. In the present study, $bla_{CTX-M-15}$ was detected in 30% (28 isolates with the gene out of 89 true ESBL-producers) of the isolates originating

from Germany, however also from the countries Kosovo, Ukraine, Croatia, and Afghanistan (S4 Table). As an additional 54% of isolates were found to carry $bla_{\text{CTX-M}}$-subtypes other than $bla_{\text{CTX-M-15}}$ (S4 Table), it may be assumed that the CTX-M beta-lactamases have a generally high prevalence, regardless of source.

A recent publication indicated large-scale transmission of hospital-associated bla_{IMP}-carrying isolates into wildlife after feeding of birds at a local waste depot [6]. But this finding could not be supported by results of a study from the same year with hardly any confirmed transmission from 22 ESBL-positive humans to their companion dogs [68]. Hypotheses regarding transmission pathways and reservoirs are often oversimplified in single studies whereas the reality is far more complex [42].

Phylogeny

The bacterial species *E. coli* possesses great genetic diversity, with >7,000 identified MLST STs [27]. Although the vast majority of *E. coli* is a prolific commensal part of the gut microbiome, selected serotypes cause serious disease, including the enterohemorrhagic or extraintestinal pathogenic *E. coli* (EHEC, ExPEC), which express various virulence and toxin genes [64, 69–70]. Outbreak investigation revealed that an epidemiological linkage was estimated if two isolates revealed the same MLST ST, and differed by less than ten core SNPs [17, 71]. In contrast, antimicrobial resistance in *E. coli* is not restricted to specific clones, as it has been identified in a broad variety of genotypes isolated from human and animal sources [11, 42, 51, 67, 70, 72]. We observed representative diversity among the 85 ESBL-producing *E. coli* characterized here, with 34 different MLST STs, including three currently unassigned STs (Fig 1 and S1 Table). Amongst the isolates belonging to MLST ST10, additional SNP diversity could be identified, likely related to the different countries of origin for these isolates (Fig 1 and S3 Table).

In the present study, four clusters (ST744, ST648, ST410, ST23) were identified that included isolates of different dog-origin that also showed similar beta-lactamase CDS profiles and lacked SNP differences (Fig 1). This suggests that these isolates epidemiologically share the same ancestor, which may be explained by mutual/ reciprocal transmission, as the dogs in question regularly share the same training facility and runout (Fig 1). Similar findings were recently published where low genetic diversity was described for 297 ST131 *E. coli* strains isolated in a longitudinal study from a group of patients living in a long-term care facility, indicating acquisition from a common source or person-to-person transmission [63].

Supporting information

S1 Table. Origin of the studied isolates, MLST ST data and virulence CDSs.
(XLSX)

S2 Table. Results of the microbouillon dilution method and interpretation of the MICs according to CLSI guidelines in green (susceptible), yellow (intermediate) and red (resistant) [21–22].
(XLSX)

S3 Table. GenBank references for the genomes analysed in the present study.
(XLSX)

S4 Table. Results of the *in silico* analysis regarding antimicrobial resistance CDSs with the large-scale blast score ratio (LS-BSR) pipeline.
(XLSX)

S5 Table. Direct comparison of the *in vitro* and *in silico* data.
(XLSX)

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Supplementary data S1 Table

Origin of the studie isolates

MLST ST data and virulence CDS

					MLST								Virulence CDS						
Isolate	Species	Country of origin	Source	Sampling date	adk	fumC	gyrB	icd	mdh	purA	recA	MLST ST	lpfA; AB161111.1	tsh; AF218073.1	argW; U11296.1	ireA; KU295572.1	estA; AF005091.1	STp; FN649417.1:c57269-57051	senB; Z54195.1
AFG_SD01_1510_Aca_091	Aeromonas caviae	Afghanistan	stray dog	Oct-15															
AFG_SD03_1510_Ahy_093	Aeromonas hydrophila	Afghanistan	stray dog	Oct-15															
GER_MD08_1505_Ecl_089	Enterobacter cloacae	Germany	military dog	13-May-15															
GER_MD12_1511_Ecl_086	Enterobacter cloacae	Germany	military dog	2-Nov-15															
GER_MD16_1505_Esp_090	Enterobacter sp.*	Germany	military dog	15-May-15															
AFG_SD04_1510_Eco_094	Escherichia coli	Afghanistan	stray dog	Oct-15	8	11	4	8	7	8	6	181	neg	neg	neg	neg	neg	neg	neg
AFG_SD05_1510_Eco_095	Escherichia coli	Afghanistan	stray dog	Oct-15	64	461	5	83	8	8	256	3586	neg	neg	neg	neg	neg	neg	neg
AFG_SD06_1510_Eco_096	Escherichia coli	Afghanistan	stray dog	Oct-15	64	461	5	83	8	8	256	3586	neg	neg	neg	neg	neg	neg	neg
AFG_SD07_1510_Eco_097	Escherichia coli	Afghanistan	stray dog	Oct-15	10	11	4	NA	8	108	352	NA	neg	neg	0.87	neg	neg	neg	neg
AFG_SD08_1510_Eco_098	Escherichia coli	Afghanistan	stray dog	Oct-15	10	11	4	NA	8	108	352	NA	neg	neg	0.87	neg	neg	neg	neg
CRO_MD01_1509_Eco_084	Escherichia coli	Croatia	military dog	10-Sep-15	8	7	1	8	8	13	6	2450	neg	neg	neg	neg	neg	neg	neg
CRO_MD02_1509_Eco_085	Escherichia coli	Croatia	military dog	10-Sep-15															
GER_CD71_1507_Eco_078	Escherichia coli	Germany	private dog, companion	20-Jul-15	6	19	3	18	9	8	6	1056	0.99	neg	neg	neg	neg	neg	neg
GER_CD71_1507_Eco_079	Escherichia coli	Germany	private dog, companion	20-Jul-15	6	19	3	18	9	8	6	1056	0.99	neg	neg	neg	neg	neg	neg
GER_CD72_1507_Eco_080	Escherichia coli	Germany	private dog, companion	20-Jul-15	6	4	4	16	24	8	14	58	neg	neg	neg	0.97	neg	neg	neg
GER_CD72_1507_Eco_081	Escherichia coli	Germany	private dog, companion	20-Jul-15	6	4	12	1	20	18	7	410	neg	neg	neg	neg	neg	neg	neg
GER_CD72_1507_Eco_082	Escherichia coli	Germany	private dog, companion	20-Jul-15	6	4	4	16	24	8	14	58	neg	neg	neg	0.97	neg	neg	neg
GER_EN01_1501_Eco_087	Escherichia coli	Germany	environment	28-Jan-15	10	11	4	8	8	8	2	10	neg	neg	neg	neg	1.0	1.0	neg
GER_EN02_1501_Eco_088	Escherichia coli	Germany	environment	28-Jan-15	49	4	44	9	11	35	7	120	neg	neg	0.71	neg	neg	neg	neg
GER_MD01_1505_Eco_001	Escherichia coli	Germany	military dog	4-May-15	10	11	4	8	8	8	2	10	neg	neg	neg	neg	neg	neg	neg
GER_MD01_1505_Eco_004	Escherichia coli	Germany	military dog	5-May-15	10	11	4	8	8	8	2	10	neg	neg	neg	neg	neg	neg	neg
GER_MD01_1505_Eco_008	Escherichia coli	Germany	military dog	6-May-15															
GER_MD01_1505_Eco_009	Escherichia coli	Germany	military dog	6-May-15	10	11	4	8	8	8	2	10	neg	neg	neg	neg	neg	neg	neg
GER_MD01_1505_Eco_047	Escherichia coli	Germany	military dog	25-May-15	43	41	15	18	11	7	6	101	0.99	neg	neg	neg	neg	neg	neg
GER_MD01_1505_Eco_048	Escherichia coli	Germany	military dog	25-May-15	43	41	15	18	11	7	6	101	0.99	neg	neg	neg	neg	neg	neg
GER_MD01_1509_Eco_054	Escherichia coli	Germany	military dog	1-Sep-15	20	45	56	106	7	50	46	1163	neg	neg	neg	neg	neg	neg	neg
GER_MD01_1509_Eco_055	Escherichia coli	Germany	military dog	1-Sep-15	20	45	56	106	7	50	46	1163	neg	neg	neg	neg	neg	neg	neg
GER_MD01_1509_Eco_056	Escherichia coli	Germany	military dog	1-Sep-15	6	11	5	8	7	8	108	868	neg	neg	0.89	neg	neg	neg	neg
GER_MD01_1509_Eco_057	Escherichia coli	Germany	military dog	1-Sep-15	6	11	5	8	7	8	108	868	neg	neg	0.89	neg	neg	neg	neg
GER_MD01_1509_Eco_058	Escherichia coli	Germany	military dog	1-Sep-15	64	7	1	1	8	8	6	398	neg	neg	neg	neg	neg	neg	neg
GER_MD01_1509_Eco_059	Escherichia coli	Germany	military dog	1-Sep-15	6	11	5	8	7	8	108	868	neg	neg	0.89	neg	neg	neg	neg
GER_MD01_1603_Eco_066	Escherichia coli	Germany	military dog	29-Mar-16	99	6	33	33	24	8	7	453	neg	neg	0.88	neg	neg	neg	neg
GER_MD02_1505_Eco_034	Escherichia coli	Germany	military dog	18-May-15	10	11	135	8	8	8	2	744	neg	neg	0.89	neg	neg	neg	neg
GER_MD02_1505_Eco_040	Escherichia coli	Germany	military dog	18-May-15	10	11	135	8	8	8	2	744	neg	neg	0.89	neg	neg	neg	neg
GER_MD02_1511_Eco_060	Escherichia coli	Germany	military dog	2-Nov-15	6	4	12	1	20	13	7	23	neg	neg	neg	neg	neg	neg	neg
GER_MD02_1511_Eco_061	Escherichia coli	Germany	military dog	2-Nov-15	6	4	12	1	20	13	7	23	neg	neg	neg	neg	neg	neg	neg
GER_MD02_1511_Eco_064	Escherichia coli	Germany	military dog	2-Nov-15	6	4	12	1	20	18	7	410	neg	neg	neg	neg	neg	neg	0.91
GER_MD03_1505_Eco_003	Escherichia coli	Germany	military dog	5-May-15	4	26	2	25	5	8	19	315	neg	neg	neg	neg	neg	neg	neg
GER_MD03_1505_Eco_005	Escherichia coli	Germany	military dog	5-May-15	4	26	2	25	5	8	19	315	neg	neg	neg	neg	neg	neg	neg
GER_MD03_1505_Eco_006	Escherichia coli	Germany	military dog	4-May-15	43	41	15	18	11	7	6	101	0.99	neg	neg	neg	neg	neg	neg
GER_MD03_1505_Eco_010	Escherichia coli	Germany	military dog	7-May-15	10	11	135	8	8	8	2	744	neg	neg	0.89	neg	neg	neg	neg
GER_MD03_1505_Eco_011	Escherichia coli	Germany	military dog	7-May-15	10	11	135	8	8	8	2	744	neg	neg	0.89	neg	neg	neg	neg
GER_MD03_1505_Eco_015	Escherichia coli	Germany	military dog	11-May-15	4	26	2	25	5	8	19	315	neg	neg	neg	neg	neg	neg	neg
GER_MD03_1505_Eco_016	Escherichia coli	Germany	military dog	11-May-15	4	26	2	25	5	8	19	315	neg	neg	neg	neg	neg	neg	neg
GER_MD03_1505_Eco_017	Escherichia coli	Germany	military dog	11-May-15	6	4	4	16	24	8	14	58	neg	neg	neg	1.0	neg	neg	neg
GER_MD03_1505_Eco_018	Escherichia coli	Germany	military dog	11-May-15	10	11	135	8	8	8	2	744	neg	neg	0.89	neg	neg	neg	neg
GER_MD03_1505_Eco_041	Escherichia coli	Germany	military dog	18-May-15	154	187	22	52	130	129	4	973	neg	neg	neg	neg	neg	neg	neg
GER_MD03_1507_Eco_050	Escherichia coli	Germany	military dog	6-Jul-15	154	187	22	52	130	129	4	973	neg	neg	neg	neg	neg	neg	neg
GER_MD06_1505_Eco_002	Escherichia coli	Germany	military dog	4-May-15	10	11	135	8	8	8	2	744	neg	neg	0.89	neg	neg	neg	neg
GER_MD06_1505_Eco_007	Escherichia coli	Germany	military dog	5-May-15	10	11	135	8	8	8	2	744	neg	neg	0.89	neg	neg	neg	neg
GER_MD06_1511_Eco_062	Escherichia coli	Germany	military dog	2-Nov-15	6	4	12	1	20	18	7	410	neg	neg	neg	neg	neg	neg	0.91
GER_MD06_1511_Eco_065	Escherichia coli	Germany	military dog	2-Nov-15	6	4	12	1	20	13	7	23	neg	neg	neg	neg	neg	neg	neg
GER_MD07_1505_Eco_012	Escherichia coli	Germany	military dog	7-May-15	38	39	30	13	17	25	28	681	0.9	neg	neg	neg	neg	neg	neg
GER_MD07_1505_Eco_013	Escherichia coli	Germany	military dog	8-May-15	38	39	30	13	17	25	28	681	0.9	neg	neg	neg	neg	neg	neg
GER_MD07_1505_Eco_014	Escherichia coli	Germany	military dog	7-May-15	38	39	30	13	17	25	28	681	0.9	neg	neg	neg	neg	neg	neg
GER_MD08_1505_Eco_019	Escherichia coli	Germany	military dog	11-May-15	6	4	12	1	20	12	7	88	neg	neg	neg	neg	neg	neg	neg
GER_MD08_1505_Eco_035	Escherichia coli	Germany	military dog	13-May-15	6	4	12	1	20	12	7	88	neg	neg	neg	neg	neg	neg	neg
GER_MD08_1505_Eco_046	Escherichia coli	Germany	military dog	13-May-15															
GER_MD11_1505_Eco_020	Escherichia coli	Germany	military dog	11-May-15	21	35	27	6	5	2	4	2459	neg	neg	0.95	neg	neg	neg	neg

Supplementary data S1 Table

Origin of the studie isolates
MLST ST data and virulence CDS

					MLST								Virulence CDS						
Isolate	Species	Country of origin	Source	Sampling date	adk	fumC	gyrB	icd	mdh	purA	recA	MLST ST	lpfA; AB161111.1	tsh; AF218073.1	argW; U11296.1	ireA; KU295572.1	estA; AF005091.1	STp; FN649417.1:c57269-57051	senB; Z54195.1
GER_MD11_1505_Eco_023	Escherichia coli	Germany	military dog	13-May-15	8	7	4	8	8	18	6	2325	neg	neg	neg	neg	neg	neg	neg
GER_MD11_1505_Eco_024	Escherichia coli	Germany	military dog	13-May-15	NA	11	4	8	8	8	2	NA	neg	neg	neg	neg	neg	neg	neg
GER_MD11_1505_Eco_025	Escherichia coli	Germany	military dog	11-May-15	6	11	4	8	8	8	2	48	neg	neg	neg	neg	neg	neg	neg
GER_MD11_1505_Eco_026	Escherichia coli	Germany	military dog	11-May-15	10	11	135	8	8	8	2	744	neg	neg	0.89	neg	neg	neg	neg
GER_MD11_1505_Eco_027	Escherichia coli	Germany	military dog	11-May-15	8	7	4	8	8	18	6	2325	neg	neg	neg	neg	neg	neg	neg
GER_MD11_1505_Eco_028	Escherichia coli	Germany	military dog	13-May-15															
GER_MD11_1505_Eco_029	Escherichia coli	Germany	military dog	13-May-15	8	7	4	8	8	18	6	2325	neg	neg	neg	neg	neg	neg	neg
GER_MD11_1505_Eco_030	Escherichia coli	Germany	military dog	13-May-15	10	11	135	8	8	8	2	744	neg	neg	0.89	neg	neg	neg	neg
GER_MD11_1505_Eco_031	Escherichia coli	Germany	military dog	13-May-15	6	NA	51	NA	1	162	42	NA	neg	neg	neg	neg	neg	neg	neg
GER_MD11_1505_Eco_032	Escherichia coli	Germany	military dog	13-May-15	6	11	4	8	8	8	2	48	neg	neg	neg	neg	neg	neg	neg
GER_MD11_1505_Eco_033	Escherichia coli	Germany	military dog	13-May-15															
GER_MD11_1505_Eco_036	Escherichia coli	Germany	military dog	15-May-15	21	35	27	6	5	2	4	2459	neg	neg	0.95	neg	neg	neg	neg
GER_MD11_1505_Eco_042	Escherichia coli	Germany	military dog	15-May-15	21	35	27	6	5	2	4	2459	neg	neg	0.95	neg	neg	neg	neg
GER_MD14_1505_Eco_037	Escherichia coli	Germany	military dog	15-May-15	92	4	87	96	70	58	2	648	neg	neg	neg	neg	neg	neg	neg
GER_MD14_1505_Eco_038	Escherichia coli	Germany	military dog	18-May-15	92	4	87	96	70	58	2	648	neg	neg	neg	neg	neg	neg	neg
GER_MD14_1505_Eco_043	Escherichia coli	Germany	military dog	15-May-15	92	4	87	96	70	58	2	648	neg	neg	neg	neg	neg	neg	neg
GER_MD14_1505_Eco_044	Escherichia coli	Germany	military dog	18-May-15	92	4	87	96	70	58	2	648	neg	neg	neg	neg	neg	neg	neg
GER_MD14_1507_Eco_051	Escherichia coli	Germany	military dog	6-Jul-15	9	23	33	1	7	8	156	1662	neg	neg	neg	neg	neg	neg	neg
GER_MD14_1507_Eco_053	Escherichia coli	Germany	military dog	6-Jul-15	9	23	33	1	7	8	156	1662	neg	neg	neg	neg	neg	neg	neg
GER_MD14_1511_Eco_063	Escherichia coli	Germany	military dog	2-Nov-15	6	4	12	1	20	8	7	90	neg	neg	neg	neg	neg	neg	neg
GER_MD15_1507_Eco_052	Escherichia coli	Germany	military dog	6-Jul-15	9	23	33	1	7	8	156	1662	neg	neg	neg	neg	neg	neg	neg
GER_MD17_1505_Eco_039	Escherichia coli	Germany	military dog	18-May-15	92	4	87	96	70	58	2	648	neg	neg	neg	neg	neg	neg	neg
GER_MD17_1505_Eco_045	Escherichia coli	Germany	military dog	18-May-15	92	4	87	96	70	58	2	648	neg	neg	neg	neg	neg	neg	neg
GER_MD67_1606_Eco_100	Escherichia coli	Germany	military dog	21-Jun-16	6	4	14	1	20	62	7	345	neg	0.8	0.87	neg	neg	neg	neg
GER_MD77_1507_Eco_075	Escherichia coli	Germany	military dog	2-Jul-15	6	4	4	16	24	8	14	58	neg	neg	neg	0.97	neg	neg	neg
GER_MD77_1507_Eco_076	Escherichia coli	Germany	military dog	2-Jul-15															
GER_MD77_1507_Eco_077	Escherichia coli	Germany	military dog	2-Jul-15	9	65	5	1	9	13	6	162	0.99	neg	neg	neg	neg	neg	neg
GER_MD90_1604_Eco_099	Escherichia coli	Germany	military dog	11-Apr-16	88	103	19	36	23	44	26	372	0.9	neg	neg	neg	neg	neg	neg
KOS_DS01_1507_Eco_070	Escherichia coli	Kosovo	shelter dog	2-Jul-15	10	11	4	8	8	8	2	10	neg	neg	0.87	neg	neg	neg	neg
KOS_DS02_1507_Eco_071	Escherichia coli	Kosovo	shelter dog	2-Jul-15	9	65	5	1	9	13	6	162	0.99	neg	neg	neg	neg	neg	neg
KOS_DS03_1507_Eco_072	Escherichia coli	Kosovo	shelter dog	2-Jul-15															
KOS_SD04_1507_Eco_073	Escherichia coli	Kosovo	stray dog	9-Jul-15	6	4	14	16	24	8	14	155	neg	neg	neg	neg	neg	neg	neg
KOS_SD05_1507_Eco_074	Escherichia coli	Kosovo	stray dog	9-Jul-15	13	14	19	36	23	11	10	127	neg	neg	neg	neg	neg	neg	0.91
KOS_SF06_1605_Eco_101	Escherichia coli	Kosovo	stray fox	15-Jun-16	10	11	4	8	8	8	2	10	neg	neg	neg	neg	neg	neg	neg
UKR_MD01_1506_Eco_067	Escherichia coli	Ukraine	military dog	5-Jun-15	10	11	4	8	8	8	2	10	neg	0.57	neg	neg	neg	neg	neg
UKR_MD01_1506_Eco_068	Escherichia coli	Ukraine	military dog	5-Jun-15	10	11	4	8	8	8	2	10	neg	0.57	neg	neg	neg	neg	neg
UKR_MD01_1506_Eco_069	Escherichia coli	Ukraine	military dog	5-Jun-15	21	35	27	6	5	5	4	69	neg	neg	neg	neg	neg	neg	0.91
GER_MD10_1505_Pmi_049	Proteus mirabilis	Germany	military dog	25-May-15															
GER_MD14_1510_Pae_083	Pseudomonas aeruginosa	Germany	military dog	6-Oct-15															
AFG_SD02_1510_Psp_092	Pseudomonas sp.*	Afghanistan	stray dog	Oct-15															

NA: not assigned
* Identification of isolates on genus level only

Supplementary data S2 Table

Microbouillon dilution results
interpretation according to CLSI guidelines

Isolate name	Species	COX	CTX	C/C	CAZ	CZC	CEP	CMC	ERT*	MER	in vitro ESBL-production
AFG_SD01_1510_Aca_091	Aeromonas caviae	S	32	16	8	1	4	1	S	S	n.d.
AFG_SD03_1510_Ahy_093	Aeromonas hydrophila	8	>128	S	4	S	64	S	S	S	n.d.
AFG_SD02_1510_Psp_092	Pseudomonas species	>32	16	16	2	2	S	1	1	S	n.d.
GER_MD12_1511_Ecl_086	Enterobacter cloacae	>32	2	16	2	16	S	S	S	S	neg
GER_MD16_1505_Esp_090	Enterobacter species	>32	4	8	4	4	S	S	S	S	neg
GER_MD07_1505_Eco_013	Escherichia coli	S	4	4	16	16	1	1	n.d.	S	neg
GER_MD07_1505_Eco_012	Escherichia coli	S	8	4	16	16	1	1	n.d.	S	neg
GER_MD90_1604_Eco_099	Escherichia coli	>32	64	>32	>128	64	8	2	S	S	neg
GER_EN01_1501_Eco_087	Escherichia coli	S	2	S	S	S	8	S	S	S	neg
GER_EN02_1501_Eco_088	Escherichia coli	>32	2	4	8	2	S	S	S	S	neg
GER_MD01_1509_Eco_059	Escherichia coli	S	S	S	S	S	S	S	S	S	neg
GER_MD14_1510_Pae_083	Pseudomonas aeruginosa	>32	32	16	S	2	S	S	1	S	neg
GER_MD08_1505_Ecl_089	Enterobacter cloacae	>32	>128	>32	64	32	4	2	S	S	pos
GER_MD01_1505_Eco_001	Escherichia coli	8	64	S	2	S	32	S	n.d.	S	pos
GER_MD01_1505_Eco_009	Escherichia coli	S	64	S	1	S	64	S	n.d.	S	pos
GER_MD03_1505_Eco_006	Escherichia coli	S	64	S	1	S	64	S	n.d.	S	pos
GER_MD03_1505_Eco_010	Escherichia coli	S	64	S	1	S	64	S	n.d.	S	pos
GER_MD01_1505_Eco_004	Escherichia coli	S	64	S	2	S	64	S	n.d.	S	pos
GER_MD03_1505_Eco_011	Escherichia coli	S	64	S	2	S	64	S	n.d.	S	pos
GER_MD06_1505_Eco_007	Escherichia coli	S	64	S	2	S	64	S	n.d.	S	pos
GER_MD06_1505_Eco_002	Escherichia coli	8	64	S	4	S	64	S	n.d.	S	pos
GER_MD03_1505_Eco_003	Escherichia coli	S	64	S	4	S	64	S	n.d.	S	pos
GER_MD03_1505_Eco_005	Escherichia coli	S	64	S	8	S	64	S	n.d.	S	pos
GER_MD01_1505_Eco_008	Escherichia coli	S	64	S	64	S	64	S	n.d.	S	pos
GER_MD07_1505_Eco_014	Escherichia coli	8	4	4	16	4	2	0.5	S	S	pos
AFG_SD07_1510_Eco_097	Escherichia coli	S	64	S	4	S	16	S	S	S	pos
GER_MD08_1505_Eco_046	Escherichia coli	>32	4	4	8	8	16	1	S	S	pos
GER_MD08_1505_Eco_035	Escherichia coli	>32	4	2	16	8	16	1	S	S	pos
GER_MD08_1505_Eco_019	Escherichia coli	>32	4	4	16	8	16	2	S	S	pos
AFG_SD08_1510_Eco_098	Escherichia coli	S	64	S	16	S	16	S	S	S	pos
GER_MD67_1606_Eco_100	Escherichia coli	8	64	S	S	S	16	S	S	S	pos
UKR_MD01_1506_Eco_068	Escherichia coli	S	64	S	4	S	32	S	S	S	pos
GER_MD03_1505_Eco_016	Escherichia coli	8	>128	S	16	S	32	S	S	S	pos
GER_MD03_1505_Eco_017	Escherichia coli	S	128	S	S	S	32	S	S	S	pos
GER_MD02_1505_Eco_034	Escherichia coli	S	>128	S	2	S	64	S	S	S	pos
GER_MD02_1505_Eco_040	Escherichia coli	S	>128	S	2	S	64	S	S	S	pos
GER_MD03_1505_Eco_018	Escherichia coli	S	>128	S	2	S	64	S	S	S	pos
GER_MD11_1505_Eco_022	Escherichia coli	S	>128	S	2	S	64	S	S	S	pos
UKR_MD01_1506_Eco_067	Escherichia coli	S	64	S	4	S	64	S	S	S	pos
GER_MD03_1505_Eco_015	Escherichia coli	8	128	S	8	S	64	S	S	S	pos
KOS_DS02_1507_Eco_071	Escherichia coli	S	128	S	S	S	64	S	S	S	pos
GER_CD72_1507_Eco_082	Escherichia coli	S	>128	S	S	S	64	S	S	S	pos
KOS_SF06_1605_Eco_101	Escherichia coli	S	>128	S	S	S	64	S	S	S	pos
GER_MD15_1507_Eco_052	Escherichia coli	8	128	S	2	S	128	S	S	S	pos
GER_MD14_1507_Eco_051	Escherichia coli	S	128	S	2	S	128	S	S	S	pos
GER_MD11_1505_Eco_030	Escherichia coli	8	>128	S	2	S	128	S	S	S	pos
GER_MD06_1511_Eco_065	Escherichia coli	S	>128	S	2	S	128	S	S	S	pos
GER_MD14_1507_Eco_053	Escherichia coli	S	>128	S	2	S	128	S	S	S	pos
GER_MD11_1505_Eco_020	Escherichia coli	S	>128	1	4	S	128	S	S	S	pos
GER_MD01_1509_Eco_058	Escherichia coli	S	>128	S	4	S	128	S	S	S	pos
GER_MD11_1505_Eco_025	Escherichia coli	S	>128	S	4	S	128	S	S	S	pos

Supplementary data S2 Table

Microbouillon dilution results
interpretation according to CLSI guidelines

Isolate name	Species	COX	CTX	C/C	CAZ	CZC	CEP	CMC	ERT*	MER	in vitro ESBL-production
GER_MD14_1511_Eco_063	Escherichia coli	S	>128	S	4	S	128	S	S	S	pos
GER_CD72_1507_Eco_080	Escherichia coli	S	>128	S	8	S	128	S	S	S	pos
GER_MD01_1509_Eco_056	Escherichia coli	S	>128	S	8	S	128	S	S	S	pos
GER_MD01_1509_Eco_057	Escherichia coli	S	>128	S	8	S	128	S	S	S	pos
KOS_SD04_1507_Eco_073	Escherichia coli	S	>128	S	32	S	128	S	S	S	pos
GER_CD71_1507_Eco_079	Escherichia coli	8	>128	S	S	S	128	S	S	S	pos
GER_CD71_1507_Eco_078	Escherichia coli	S	>128	S	S	S	128	S	S	S	pos
GER_MD02_1511_Eco_060	Escherichia coli	S	>128	S	2	S	>128	S	S	S	pos
GER_MD11_1505_Eco_023	Escherichia coli	S	>128	S	2	0.5	>128	S	S	S	pos
GER_MD11_1505_Eco_028	Escherichia coli	S	>128	S	2	0.5	>128	S	S	S	pos
GER_MD77_1507_Eco_077	Escherichia coli	S	>128	S	2	S	>128	S	S	S	pos
GER_MD11_1505_Eco_024	Escherichia coli	S	>128	0.5	4	S	>128	S	S	S	pos
GER_MD03_1505_Eco_041	Escherichia coli	8	>128	S	4	S	>128	S	S	S	pos
GER_MD03_1507_Eco_050	Escherichia coli	8	>128	S	4	0.5	>128	S	S	S	pos
GER_MD02_1511_Eco_061	Escherichia coli	S	>128	S	4	S	>128	S	S	S	pos
GER_MD11_1505_Eco_026	Escherichia coli	S	>128	S	4	0.5	>128	S	S	S	pos
GER_MD11_1505_Eco_027	Escherichia coli	S	>128	S	4	0.5	>128	S	S	S	pos
GER_MD11_1505_Eco_029	Escherichia coli	S	>128	S	4	S	>128	S	S	S	pos
GER_MD11_1505_Eco_032	Escherichia coli	S	>128	S	4	S	>128	S	S	S	pos
GER_MD11_1505_Eco_042	Escherichia coli	S	>128	S	4	0.5	>128	S	S	S	pos
GER_MD01_1509_Eco_054	Escherichia coli	S	>128	S	8	0.5	>128	S	S	S	pos
GER_MD01_1509_Eco_055	Escherichia coli	S	>128	S	8	0.5	>128	S	S	S	pos
GER_MD11_1505_Eco_033	Escherichia coli	S	>128	S	8	1	>128	0.5	S	S	pos
GER_MD77_1507_Eco_076	Escherichia coli	8	>128	S	16	S	>128	S	S	S	pos
KOS_DS01_1507_Eco_070	Escherichia coli	8	>128	S	16	S	>128	S	S	S	pos
GER_MD11_1505_Eco_021	Escherichia coli	S	>128	S	16	S	>128	S	S	S	pos
KOS_DS03_1507_Eco_072	Escherichia coli	S	>128	S	16	S	>128	S	S	S	pos
KOS_SD05_1507_Eco_074	Escherichia coli	S	>128	S	16	S	>128	S	S	S	pos
UKR_MD01_1506_Eco_069	Escherichia coli	S	>128	S	16	S	>128	S	S	S	pos
GER_MD14_1505_Eco_037	Escherichia coli	32	>128	0.5	32	0.5	>128	S	S	S	pos
GER_MD17_1505_Eco_039	Escherichia coli	32	>128	0.5	32	S	>128	0.5	S	S	pos
AFG_SD04_1510_Eco_094	Escherichia coli	8	>128	S	32	S	>128	S	S	S	pos
GER_MD01_1505_Eco_047	Escherichia coli	8	>128	S	32	S	>128	0.5	S	S	pos
GER_MD14_1505_Eco_044	Escherichia coli	32	>128	S	32	S	>128	2	S	S	pos
GER_MD17_1505_Eco_045	Escherichia coli	32	>128	S	32	S	>128	2	S	S	pos
AFG_SD05_1510_Eco_095	Escherichia coli	S	>128	S	32	S	>128	S	S	S	pos
AFG_SD06_1510_Eco_096	Escherichia coli	S	>128	S	32	S	>128	S	S	S	pos
GER_MD02_1511_Eco_064	Escherichia coli	S	>128	S	32	S	>128	S	S	S	pos
GER_MD06_1511_Eco_062	Escherichia coli	S	>128	S	32	S	>128	S	S	S	pos
GER_MD14_1505_Eco_038	Escherichia coli	32	>128	0.5	64	1	>128	0.5	S	S	pos
GER_MD14_1505_Eco_043	Escherichia coli	32	>128	0.5	64	4	>128	0.5	S	S	pos
GER_MD01_1505_Eco_048	Escherichia coli	8	>128	S	64	S	>128	S	S	S	pos
GER_CD72_1507_Eco_081	Escherichia coli	S	>128	S	64	S	>128	S	S	S	pos
GER_MD01_1603_Eco_066	Escherichia coli	S	>128	S	64	S	>128	S	S	S	pos
GER_MD77_1507_Eco_075	Escherichia coli	S	>128	S	64	S	>128	S	S	S	pos
CRO_MD01_1509_Eco_084	Escherichia coli	>32	>128	2	>128	1	>128	1	S	S	pos
CRO_MD02_1509_Eco_085	Escherichia coli	>32	>128	2	>128	2	>128	2	S	S	pos
GER_MD11_1505_Eco_031	Escherichia coli	S	8	0.5	S	S	>128	2	S	S	pos
GER_MD10_1505_Pmi_049	Proteus mirabilis	8	>128	S	4	S	>128	S	S	S	pos

*design of Merlin micronaut plates was changed during study VI>VII
n.d.: not determined
the combination of CTX, CAZ or CEP with clavulainc acid was tested in a standard dilution of 4 µg/ ml clavulanic acid for each of the antibiotics

Supplementary data S3 Table GenBank references

Sample Name	BioSample	SRA_Run	Assembly_Accession
AFG_SD01_1510_Aca_091	SAMN08519207	SRR6724101	PUTR00000000
AFG_SD03_1510_Ahy_093	SAMN08519208	SRR6724100	PUTQ00000000
GER_MD01_1505_Eco_001	SAMN08519209	SRR6724103	PUTP00000000
GER_MD06_1505_Eco_002	SAMN08519210	SRR6724102	PUTO00000000
GER_MD03_1505_Eco_003	SAMN08519211	SRR6724105	PUTN00000000
GER_MD01_1505_Eco_004	SAMN08519212	SRR6724104	PUTM00000000
GER_MD03_1505_Eco_005	SAMN08519213	SRR6724107	PUTL00000000
GER_MD03_1505_Eco_006	SAMN08519214	SRR6724106	PUTK00000000
GER_MD06_1505_Eco_007	SAMN08519215	SRR6724109	PUTJ00000000
GER_MD01_1505_Eco_009	SAMN08519216	SRR6724108	PUTI00000000
GER_MD03_1505_Eco_010	SAMN08519217	SRR6724075	PUTH00000000
GER_MD03_1505_Eco_011	SAMN08519218	SRR6724074	PUTG00000000
GER_MD07_1505_Eco_012	SAMN08519219	SRR6724073	PUTF00000000
GER_MD07_1505_Eco_013	SAMN08519220	SRR6724072	PUTE00000000
GER_MD07_1505_Eco_014	SAMN08519221	SRR6724079	PUTD00000000
GER_MD03_1505_Eco_015	SAMN08519222	SRR6724078	PUTC00000000
GER_MD03_1505_Eco_016	SAMN08519223	SRR6724077	PUTB00000000
GER_MD03_1505_Eco_017	SAMN08519224	SRR6724076	PUTA00000000
GER_MD03_1505_Eco_018	SAMN08519225	SRR6724071	PUSZ00000000
GER_MD08_1505_Eco_019	SAMN08519226	SRR6724070	PUSY00000000
GER_MD11_1505_Eco_020	SAMN08519227	SRR6724112	PUSX00000000
GER_MD11_1505_Eco_021	SAMN08519228	SRR6724113	PUSW00000000
GER_MD11_1505_Eco_023	SAMN08519229	SRR6724110	PUSV00000000
GER_MD11_1505_Eco_024	SAMN08519230	SRR6724111	PUSU00000000
GER_MD11_1505_Eco_025	SAMN08519231	SRR6724116	PUST00000000
GER_MD11_1505_Eco_026	SAMN08519232	SRR6724117	PUSS00000000
GER_MD11_1505_Eco_027	SAMN08519233	SRR6724114	PUSR00000000
GER_MD11_1505_Eco_029	SAMN08519234	SRR6724115	PUSQ00000000
GER_MD11_1505_Eco_030	SAMN08519235	SRR6724118	PUSP00000000
GER_MD11_1505_Eco_031	SAMN08519236	SRR6724119	PUSO00000000
GER_MD11_1505_Eco_032	SAMN08519237	SRR6724081	PUSN00000000
GER_MD02_1505_Eco_034	SAMN08519238	SRR6724080	PUSM00000000
GER_MD08_1505_Eco_035	SAMN08519239	SRR6724083	PUSL00000000
GER_MD11_1505_Eco_036	SAMN08519240	SRR6724082	PUSK00000000
GER_MD14_1505_Eco_037	SAMN08519241	SRR6724085	PUSJ00000000
GER_MD14_1505_Eco_038	SAMN08519242	SRR6724084	PUSI00000000
GER_MD17_1505_Eco_039	SAMN08519243	SRR6724087	PUSH00000000
GER_MD02_1505_Eco_040	SAMN08519244	SRR6724086	PUSG00000000
GER_MD03_1505_Eco_041	SAMN08519245	SRR6724089	PUSF00000000
GER_MD11_1505_Eco_042	SAMN08519246	SRR6724088	PUSE00000000
GER_MD14_1505_Eco_043	SAMN08519247	SRR6724027	PUSD00000000
GER_MD14_1505_Eco_044	SAMN08519248	SRR6724028	PUSC00000000
GER_MD17_1505_Eco_045	SAMN08519249	SRR6724029	PUSB00000000
GER_MD01_1505_Eco_047	SAMN08519250	SRR6724030	PUSA00000000
GER_MD01_1505_Eco_048	SAMN08519251	SRR6724031	PURZ00000000
GER_MD10_1505_Pmi_049	SAMN08519252	SRR6724032	PURY00000000
GER_MD03_1507_Eco_050	SAMN08519253	SRR6724033	PURX00000000
GER_MD14_1507_Eco_051	SAMN08519254	SRR6724034	PURW00000000

Supplementary data S3 Table GenBank references

Sample Name	BioSample	SRA_Run	Assembly_Accession
GER_MD15_1507_Eco_052	SAMN08519255	SRR6724035	PURV00000000
GER_MD14_1507_Eco_053	SAMN08519256	SRR6724036	PURU00000000
GER_MD01_1509_Eco_054	SAMN08519257	SRR6724097	PURT00000000
GER_MD01_1509_Eco_055	SAMN08519258	SRR6724096	PURS00000000
GER_MD01_1509_Eco_056	SAMN08519259	SRR6724095	PURR00000000
GER_MD01_1509_Eco_057	SAMN08519260	SRR6724094	PURQ00000000
GER_MD01_1509_Eco_058	SAMN08519261	SRR6724093	PURP00000000
GER_MD01_1509_Eco_059	SAMN08519262	SRR6724092	PURO00000000
GER_MD02_1511_Eco_060	SAMN08519263	SRR6724091	PURN00000000
GER_MD02_1511_Eco_061	SAMN08519264	SRR6724090	PURM00000000
GER_MD06_1511_Eco_062	SAMN08519265	SRR6724099	PURL00000000
GER_MD14_1511_Eco_063	SAMN08519266	SRR6724098	PURK00000000
GER_MD02_1511_Eco_064	SAMN08519267	SRR6724058	PURJ00000000
GER_MD06_1511_Eco_065	SAMN08519268	SRR6724059	PURI00000000
GER_MD01_1603_Eco_066	SAMN08519269	SRR6724056	PURH00000000
UKR_MD01_1506_Eco_067	SAMN08519270	SRR6724057	PURG00000000
UKR_MD01_1506_Eco_068	SAMN08519271	SRR6724054	PURF00000000
UKR_MD01_1506_Eco_069	SAMN08519272	SRR6724055	PURE00000000
KOS_DS01_1507_Eco_070	SAMN08519273	SRR6724052	PURD00000000
KOS_DS02_1507_Eco_071	SAMN08519274	SRR6724053	PURC00000000
KOS_SD04_1507_Eco_073	SAMN08519275	SRR6724050	PURB00000000
KOS_SD05_1507_Eco_074	SAMN08519276	SRR6724051	PURA00000000
GER_MD77_1507_Eco_075	SAMN08519277	SRR6724044	PUQZ00000000
GER_MD77_1507_Eco_077	SAMN08519278	SRR6724043	PUQY00000000
GER_CD71_1507_Eco_078	SAMN08519279	SRR6724046	PUQX00000000
GER_CD71_1507_Eco_079	SAMN08519280	SRR6724045	PUQW00000000
GER_CD72_1507_Eco_080	SAMN08519281	SRR6724040	PUQV00000000
GER_CD72_1507_Eco_081	SAMN08519282	SRR6724039	PUQU00000000
GER_CD72_1507_Eco_082	SAMN08519283	SRR6724042	PUQT00000000
CRO_MD01_1509_Eco_084	SAMN08519284	SRR6724041	PUQS00000000
GER_EN01_1501_Eco_087	SAMN08519285	SRR6724038	PUQR00000000
GER_EN02_1501_Eco_088	SAMN08519286	SRR6724037	PUQQ00000000
AFG_SD04_1510_Eco_094	SAMN08519287	SRR6724066	PUQP00000000
AFG_SD05_1510_Eco_095	SAMN08519288	SRR6724067	PUQO00000000
AFG_SD06_1510_Eco_096	SAMN08519289	SRR6724068	PUQN00000000
AFG_SD07_1510_Eco_097	SAMN08519290	SRR6724069	PUQM00000000
AFG_SD08_1510_Eco_098	SAMN08519291	SRR6724062	PUQL00000000
GER_MD90_1604_Eco_099	SAMN08519292	SRR6724063	PUQK00000000
GER_MD67_1606_Eco_100	SAMN08519293	SRR6724064	PUQJ00000000
KOS_SF06_1605_Eco_101	SAMN08519294	SRR6724065	PUQI00000000
GER_MD12_1511_Ecl_086	SAMN08519295	SRR6724060	PUQH00000000
GER_MD08_1505_Ecl_089	SAMN08519296	SRR6724061	PUQG00000000
GER_MD16_1505_Esp_090	SAMN08519297	SRR6724049	PUQF00000000
GER_MD14_1510_Pae_083	SAMN08519298	SRR6724048	PUQE00000000
AFG_SD02_1510_Psp_092	SAMN08519299	SRR6724047	PUQD00000000

Supplementary data S4 Table (excerpt)

in silico analysis regarding antimicrobial resistance CDSs with LS-BSR pipline

			Ambler class A, penicillinase									
Isolate	Species	ESBL-producer in vitro	blaCTX-M-1_6	blaCTX-M-2_1	blaCTX-M-3_2	blaCTX-M-14_1	blaCTX-M-15_23	blaSFO-1_1	blaSHV-12_1	blaTEM-1A_4	blaTEM-1B_1
APG_RS01_1510_Aca_091	Aeromonas caviae	negative	-	-	-	-	-	-	-	-	-
APG_RS03_1510_Ahy_093	Aeromonas hydrophila	negative	-	-	-	-	-	100.00	-	-	-
APG_RS04_1510_Eco_094	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	100.00
APG_RS05_1510_Eco_095	Escherichia coli	positive	-	-	-	-	100.00	-	-	100.00	-
APG_RS06_1510_Eco_096	Escherichia coli	positive	-	-	-	-	100.00	-	-	100.00	-
APG_RS07_1510_Eco_097	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	100.00
APG_RS09_1510_Eco_098	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	100.00
CRO_MD01_1509_Eco_084	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_CD71_1507_Eco_078	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_CD71_1507_Eco_079	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_CD72_1507_Eco_080	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_CD72_1507_Eco_081	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_CD72_1507_Eco_082	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_EN01_1501_Eco_087	Escherichia coli	negative	-	-	-	-	-	-	-	-	-
GER_EN02_1501_Eco_088	Escherichia coli	negative	-	-	-	-	-	-	-	-	-
GER_MD01_1505_Eco_001	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD01_1505_Eco_004	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MH04_1505_Eco_007	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD01_1505_Eco_047	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD01_1505_Eco_048	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD01_1509_Eco_054	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD01_1509_Eco_055	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD01_1509_Eco_056	Escherichia coli	positive	-	-	-	-	-	-	-	-	100.00
GER_MD01_1509_Eco_057	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD01_1509_Eco_058	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD01_1509_Eco_059	Escherichia coli	negative	-	-	-	-	-	-	-	-	100.00
GER_MD01_1603_Eco_064	Escherichia coli	positive	-	-	-	100.00	-	-	100.00	-	-
GER_MD02_1505_Eco_034	Escherichia coli	positive	-	-	-	100.00	-	-	-	-	100.00
GER_MD02_1505_Eco_040	Escherichia coli	positive	-	-	-	100.00	-	-	-	-	100.00
GER_MD02_1511_Eco_060	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD02_1511_Eco_061	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD02_1511_Eco_064	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD03_1505_Eco_003	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD03_1505_Eco_005	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD03_1505_Eco_006	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD03_1505_Eco_010	Escherichia coli	positive	-	-	-	100.00	-	-	-	-	100.00
GER_MD03_1505_Eco_011	Escherichia coli	positive	-	-	-	100.00	-	-	-	-	100.00
GER_MD03_1505_Eco_013	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD03_1505_Eco_014	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD03_1505_Eco_017	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD03_1505_Eco_018	Escherichia coli	positive	-	-	-	100.00	-	-	-	-	100.00
GER_MD03_1505_Eco_041	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD03_1507_Eco_050	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD04_1505_Eco_002	Escherichia coli	positive	-	-	-	100.00	-	-	-	-	100.00
GER_MD04_1505_Eco_007	Escherichia coli	positive	-	-	-	100.00	-	-	-	-	100.00
GER_MD04_1511_Eco_062	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD04_1511_Eco_065	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD07_1505_Eco_012	Escherichia coli	negative	-	-	-	-	-	-	-	-	-
GER_MD07_1505_Eco_013	Escherichia coli	negative	-	-	-	-	-	-	-	-	-
GER_MD07_1505_Eco_014	Escherichia coli	positive	-	-	-	-	-	-	-	-	-
GER_MD08_1505_Ecl_069	Enterobacter cloacae	positive	-	-	-	-	-	-	-	-	-
GER_MD08_1505_Eco_019	Escherichia coli	positive	-	-	-	-	-	-	-	-	-
GER_MD08_1505_Eco_035	Escherichia coli	positive	-	-	-	-	-	-	-	-	-
GER_MD10_1505_Pmi_049	Proteus mirabilis	positive	-	-	-	-	-	-	-	-	-
GER_MD11_1505_Eco_020	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD11_1505_Eco_021	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD11_1505_Eco_023	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD11_1505_Eco_024	Escherichia coli	positive	-	100.00	-	-	-	-	-	-	100.00
GER_MD11_1505_Eco_025	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD11_1505_Eco_026	Escherichia coli	positive	100.00	-	-	100.00	-	-	-	-	100.00
GER_MD11_1505_Eco_027	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD11_1505_Eco_029	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD11_1505_Eco_030	Escherichia coli	positive	-	-	-	100.00	-	-	-	-	100.00
GER_MD11_1505_Eco_031	Escherichia coli	positive	-	-	-	-	-	-	-	-	-
GER_MD11_1505_Eco_032	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD11_1505_Eco_036	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD11_1505_Eco_042	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD12_1511_Ecl_084	Enterobacter cloacae	negative	-	-	-	-	-	-	-	-	-
GER_MD14_1505_Eco_037	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD14_1505_Eco_038	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD14_1505_Eco_043	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD14_1505_Eco_044	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD14_1507_Eco_051	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD14_1507_Eco_053	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD14_1510_Pae_083	Pseudomonas aeruginosa	negative	-	-	-	-	-	-	-	-	-
GER_MD14_1511_Eco_063	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD15_1507_Eco_052	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	-
GER_MD16_1505_Esp_090	Enterobacter species	negative	-	-	-	-	-	-	-	-	-
GER_MD17_1505_Eco_039	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD17_1505_Eco_045	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
GER_MD47_1606_Eco_100	Escherichia coli	positive	-	-	100.00	-	-	-	-	-	100.00
GER_MD79_1507_Eco_075	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	100.00
GER_MD77_1507_Eco_077	Escherichia coli	positive	100.00	-	-	-	-	-	-	-	100.00
GER_MD90_1604_Eco_099	Escherichia coli	negative	-	-	-	-	-	-	-	-	-
ROE_DA01_1507_Eco_070	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	100.00
ROE_DA02_1507_Eco_071	Escherichia coli	positive	-	-	100.00	-	-	-	-	-	100.00
ROE_RD04_1507_Eco_073	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	100.00
ROE_RD05_1507_Eco_074	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
ROE_RP04_1605_Eco_101	Escherichia coli	positive	-	-	-	100.00	-	-	-	-	-
UKR_MD01_1506_Eco_067	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
UKR_MD01_1506_Eco_068	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	-
UKR_MD01_1506_Eco_069	Escherichia coli	positive	-	-	-	-	100.00	-	-	-	100.00

Supplementary data S4 (excerpt)

In silico analysis regarding antimicrobial resistance CDSs with LS-BSR pipline

			Ambler class C, cephalosporinase									Ambler class D		
Isolate	Species	ESBL-producer in vitro	blaACC-1_2	blaACT-7_1	blaACT-14_1	blaCMY-2_1	blaMIR-6_1	blaMOX-5_1	blaMOX-6_1	blaPAO_1	ampH_1	blaOXA-1_1	blaOXA-60_3	blaOXA-604_1
APG_BD01_1510_Aca_091	Aeromonas caviae	negative							99.91					100.00
APG_BD03_1510_Ahy_093	Aeromonas hydrophila	negative						91.84			99.75			
APG_BD04_1510_Eco_094	Escherichia coli	positive												
APG_BD05_1510_Eco_095	Escherichia coli	positive												
APG_BD06_1510_Eco_096	Escherichia coli	positive												
APG_BD07_1510_Eco_097	Escherichia coli	positive												
APG_BD08_1510_Eco_098	Escherichia coli	positive												
CRO_MD01_1509_Eco_084	Escherichia coli	positive										100.00		
GER_CD71_1507_Eco_078	Escherichia coli	positive												
GER_CD71_1507_Eco_079	Escherichia coli	positive												
GER_CD72_1507_Eco_080	Escherichia coli	positive												
GER_CD72_1507_Eco_081	Escherichia coli	positive										100.00		
GER_CD72_1507_Eco_082	Escherichia coli	positive												
GER_EN01_1501_Eco_087	Escherichia coli	negative										100.00		
GER_EN02_1501_Eco_088	Escherichia coli	negative												
GER_MD01_1505_Eco_001	Escherichia coli	positive												
GER_MD01_1505_Eco_004	Escherichia coli	positive												
GER_MD01_1505_Eco_009	Escherichia coli	positive												
GER_MD01_1505_Eco_047	Escherichia coli	positive										100.00		
GER_MD01_1505_Eco_048	Escherichia coli	positive										100.00		
GER_MD01_1509_Eco_054	Escherichia coli	positive												
GER_MD01_1509_Eco_055	Escherichia coli	positive												
GER_MD01_1509_Eco_056	Escherichia coli	positive												
GER_MD01_1509_Eco_057	Escherichia coli	positive												
GER_MD01_1509_Eco_058	Escherichia coli	positive												
GER_MD01_1509_Eco_059	Escherichia coli	negative												
GER_MD01_1603_Eco_066	Escherichia coli	positive												
GER_MD02_1505_Eco_034	Escherichia coli	positive												
GER_MD02_1505_Eco_040	Escherichia coli	positive												
GER_MD02_1511_Eco_060	Escherichia coli	positive												
GER_MD02_1511_Eco_061	Escherichia coli	positive												
GER_MD02_1511_Eco_064	Escherichia coli	positive										100.00		
GER_MD03_1505_Eco_003	Escherichia coli	positive												
GER_MD03_1505_Eco_005	Escherichia coli	positive												
GER_MD03_1505_Eco_006	Escherichia coli	positive												
GER_MD03_1505_Eco_010	Escherichia coli	positive												
GER_MD03_1505_Eco_011	Escherichia coli	positive												
GER_MD03_1505_Eco_015	Escherichia coli	positive												
GER_MD03_1505_Eco_016	Escherichia coli	positive												
GER_MD03_1505_Eco_017	Escherichia coli	positive												
GER_MD03_1505_Eco_018	Escherichia coli	positive												
GER_MD03_1505_Eco_041	Escherichia coli	positive												
GER_MD03_1507_Eco_050	Escherichia coli	positive												
GER_MD06_1505_Eco_002	Escherichia coli	positive												
GER_MD06_1505_Eco_007	Escherichia coli	positive												
GER_MD06_1511_Eco_062	Escherichia coli	positive										100.00		
GER_MD06_1511_Eco_065	Escherichia coli	positive												
GER_MD07_1505_Eco_012	Escherichia coli	negative	100.00											
GER_MD07_1505_Eco_013	Escherichia coli	negative	100.00											
GER_MD07_1505_Eco_014	Escherichia coli	positive	100.00											
GER_MD08_1505_Ec1_089	Enterobacter cloacae	positive			100.00									
GER_MD08_1505_Eco_019	Escherichia coli	positive										100.00		
GER_MD08_1505_Eco_035	Escherichia coli	positive										100.00		
GER_MD10_1505_Pmi_049	Proteus mirabilis	positive												
GER_MD11_1505_Eco_020	Escherichia coli	positive												
GER_MD11_1505_Eco_021	Escherichia coli	positive												
GER_MD11_1505_Eco_023	Escherichia coli	positive												
GER_MD11_1505_Eco_024	Escherichia coli	positive												
GER_MD11_1505_Eco_025	Escherichia coli	positive												
GER_MD11_1505_Eco_026	Escherichia coli	positive												
GER_MD11_1505_Eco_027	Escherichia coli	positive												
GER_MD11_1505_Eco_029	Escherichia coli	positive												
GER_MD11_1505_Eco_030	Escherichia coli	positive												
GER_MD11_1505_Eco_031	Escherichia coli	positive										100.00		
GER_MD11_1505_Eco_032	Escherichia coli	positive												
GER_MD11_1505_Eco_036	Escherichia coli	positive												
GER_MD11_1505_Eco_042	Escherichia coli	positive												
GER_MD12_1511_Ec1_086	Enterobacter cloacae	negative		100.00										
GER_MD14_1505_Eco_037	Escherichia coli	positive										100.00		
GER_MD14_1505_Eco_038	Escherichia coli	positive										100.00		
GER_MD14_1505_Eco_043	Escherichia coli	positive										100.00		
GER_MD14_1505_Eco_044	Escherichia coli	positive										100.00		
GER_MD14_1507_Eco_051	Escherichia coli	positive												
GER_MD14_1507_Eco_053	Escherichia coli	positive												
GER_MD14_1510_Pae_083	Pseudomonas aeruginosa	negative								100.00			100.00	
GER_MD14_1511_Eco_063	Escherichia coli	positive										100.00		
GER_MD15_1507_Eco_052	Escherichia coli	positive												
GER_MD16_1505_Esp_090	Enterobacter species	negative					100.00							
GER_MD17_1505_Eco_039	Escherichia coli	positive										100.00		
GER_MD17_1505_Eco_045	Escherichia coli	positive										100.00		
GER_MD67_1606_Eco_100	Escherichia coli	positive												
GER_MD77_1507_Eco_075	Escherichia coli	positive												
GER_MD77_1507_Eco_077	Escherichia coli	positive												
GER_MD90_1604_Eco_099	Escherichia coli	negative				100.00								
KOE_DS01_1507_Eco_070	Escherichia coli	positive												
KOE_DS02_1507_Eco_071	Escherichia coli	positive												
KOE_DS04_1507_Eco_073	Escherichia coli	positive												
KOE_DS05_1507_Eco_074	Escherichia coli	positive										100.00		
KOE_DY06_1605_Eco_101	Escherichia coli	positive												
UKR_MD01_1506_Eco_067	Escherichia coli	positive												
UKR_MD01_1506_Eco_068	Escherichia coli	positive												
UKR_MD01_1506_Eco_069	Escherichia coli	positive												

Supplementary data Table S5
(excerpt, 4 examples)

Direct comparison of the
in vitro and in silico data

Isolate	COX	CTX	C/C	CAZ	CZC	CEP	CMC	CLSI definition ESBL-producer in vitro conditions	total of ESBL CDSs in silico	blaCTX-M-1_6	blaCTX-M-2_1	blaCTX-M-3_2	blaCTX-M-14_1	blaCTX-M-15_23	blaSFO-1_1	blaSHV-12_1	blaTEM-1A_4	blaTEM-1B_1
GER_MD11_1505_Eco_026	S	>128	S	4	0.5	>128	S	pos	3	100.00	.	.	100.00	.	.	.	.	100.00
GER_MD14_1511_Eco_063	S	>128	S	4	S	128	S	pos	3	100.00	.	.	.	.	.	.	.	100.00

cphA1_1	blaVIM-1_1	blaACC-1_2	blaACT-7_1	blaACT-14_1	blaOXA-1_1	blaOXA-50_3	blaOXA-504_1
.	.	.	.	.	.	.	.
.	.	.	.	.	100.00	.	.

Isolate	COX	CTX	C/C	CAZ	CZC	CEP	CMC	CLSI definition ESBL-producer in vitro conditions	total of ESBL CDSs in silico	blaCTX-M-1_6	blaCTX-M-2_1	blaCTX-M-3_2	blaCTX-M-14_1	blaCTX-M-15_23	blaSFO-1_1	blaSHV-12_1	blaTEM-1A_4	blaTEM-1B_1
GER_MD07_1505_Eco_014	8	4	4	16	4	2	0.5	pos	2	.	.	.	.	.	.	.	.	.
GER_MD07_1505_Eco_013	S	4	4	16	16	1	1	neg	2	.	.	.	.	.	.	.	.	.

cphA1_1	blaVIM-1_1	blaACC-1_2	blaACT-7_1	blaACT-14_1	blaOXA-1_1	blaOXA-50_3	blaOXA-504_1
.	100.00	100.00	.	.	.	.	.
.	100.00	100.00	.	.	.	.	.

IV. Discussion

Today the broad field usage of antimicrobials and the associated concerns regarding antimicrobial resistance is discussed worldwide [7, 62]. The trend to counteract the increasing antimicrobial resistance has led to several actions in recent years. National committees publish guidelines for the prudent use of antibiotic pharmaceuticals [19]. One milestone was the enforcement of the national German veterinary pharmacy regulation law with rules for the application of antibiotics to animals [63]. According to this law, the resistance patterns of bacterial isolates must be determined in case of repeated or change of medication, rededication, or regarding therapy of flocks or regarding animals bred for specific purposes. De jure veterinarians must not rededicate some so called critical antibiotics, e.g. flourochinolones, 3rd and 4th generation cephalosporins. This enforcement was aimed at a reduction of the use of antibiotics, but as well as at avoiding an increase of antimicrobial resistance through non-suitable therapy. Since 2014, the amounts and application of antibiotics in animal husbandry in Germany are officially collected in a large database [36]. This measure is to monitor the overall tendency of consumption and to countersteer undesirable trends. According to DART, an affiliation of german stakeholders in the health sector, the amount of antimicrobial drugs in outpatient care, which is the biggest part of usage in human medicine remains unchanged since 2007. Participants of DART are federal authorities for example the Federal Ministry of Health and non governmental organisations, for example the German Society for Hygiene and Microbiology [64]. In veterinary medicine huge efforts lead to success and the quantum of delivered antimicrobial drugs has been reduced from 1238 metric tons in 2014 to 733 metric tons in 2017, a reducement of about 40 % [65].

To measure microbial resistance, to standardize the classification of a bacterial isolate, and to determine the therapy of a patient, the MIC was determined and evaluated into the S-I-R status for many bacterial species by the EUCAST (compare 3.) [17]. The microorganisms studied in the present work were the ESBL-producing microorganisms selected from dog feces via simple selective nutrition media [36]. The *in vitro* analyses were carried out using commercially available test system for the microbouillon dilution method. To analyze the results, standard guidelines were applied. The CLSI provided standards for *E. coli* and *Proteus*

mirabilis (the latter only when clinically relevant). There was no recommendation for *Pseudomonas aeruginosa* from the CLSI, although it is a highly relevant pathogen in human and veterinarian diagnostics [1]. The European equivalent to CLSI is the EUCAST. This committee published guidelines for Enterobacteriaceae and *Pseudomonas* spp. tested on various ESBL-effective drugs [17]. Comparing the two different guidelines we found that the EUCAST's criteria were stricter (Table 2). Consequently, isolate GER_EN01_1501_Eco_087 initially classified with an intermediate status was found to be of resistant character when applying EUCAST guidelines (Table 3).

Table 2: Breakpoints for minimal inhibitory concentrations (MIC) in mg/L for selected Enterobacteriaceae[§], with S (susceptible) and R (resistant)

Antimicrobial agent	**CLSI**			**EUCAST**	
	criteria human isolates		**veterinary isolates****		
	S ≤	**R >**		**S ≤**	**R >**
2nd generation Cephalosporin					
Cefoxitin (COX)	8	32		na	na
3rd generation Cephalosporin					
Ceftazidime (CAZ)	4	16	0,25 - 128	1	4
Ceftazidime/ Clavulanic acid (CZC)*[%]			0,25/4 - 128/4	8	8
Cefotaxime (CTX)	1	4	0,25 - 64	1	2
Cefotaxime/Clavulanic acid (C/C)			0,25/4 - 64/4		
4th generation Cephalosporin					
Cefepime (CEP)	2	16		1	4
Cefepime/ Clavulanic acid (CMC)					
Carbapenem					
Ertapenem (ERT)	0,5	2		0,5	1
Meropenem (MER)				2	8

§ *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis* (only if clinically relevant)

* EUCAST listed avibactam (4mg/L) instead of clavulanic acid

** both 3rd generation cephalosporins have to be tested

% ESBL: a $\geq$ 3 twofold concentration decrease in an MIC for either antimicrobial agent tested in combination with clavulanic acid vs the MIC of the agent when tested alone [31]

na not applicable

Furthermore, the EUCAST guidelines contained precise references for 24 individual bacteria species, and several bacterial groups, amongst are the *Burkholderia cepacia*-complex, Enterobacteriaceae, or the *viridans* group-streptococci [17]. The American CLSI structured the published guidelines as well according to specific microorganisms, but as well according to its clinical implications. For example, recommendations were published for *E. coli* isolated from the urinary tract during an infection [15-16].

Both guidelines include warnings regarding some *in vitro* susceptibility of certain species, but may be at the same time non-effective in a patient [15-17]. The CLSI published separate guidelines regarding bacteria that are relevant in veterinary medicine [15-16]. In 2015, a sub-committee for Veterinary Antimicrobial Susceptibility Testing (VetCAST) of the EUCAST was established [66]. However, there were no specific guidelines published yet.

Table 3: Assignment of bacterial isolates according to different guidelines; S (susceptible), I (intermediate), R (resistant), na (not assigned).

	COX	**CTX**	**CAZ**	**CEP**
In vitro MIC of isolate GER_EN01_1501_Eco_087	S	2	S	8
Assignment according to CLSI [15]	S	I	S	I
Assignment according to EUCAST [17]	na	I	S	**R**

Various studies revealed that inoculum effects and *in vitro* conditions may easily affect the results of MIC during antibiotic susceptibility testing. In the present study we used a densitometer (DENSIMAT, Fa. BioMerieux Biotechnologies) to make certain, that inoculum effects were excluded. As well transferring the *in vitro* results to the dosage of drugs for patients even might potentise the error regarding effective treatment [67-69]. Therefore, and to enhance the detection of known resistance, the interpretive criteria of MIC regarding most beta-lactam antibiotics, including extended-spectrum cephalosporins among Enterobacteriaceae were enforced in the past years [70]. The interpretation criteria published by the EUCAST are even stricter, and the MIC values lower, than the comparable values in both of the guidelines of the American CLSI, with human or veterinarian focus (Table 2) [15-17]. For example, cefepime is a 4th generation cephalosporin, and limited for use in humans.

However, 89% of the dog isolates in the present study were resistant according to CLSI interpretation [36]. Considering the EUCAST guidelines even 93% of these isolates are considered as resistant to cefepime, including most of the stray dog isolates [17, 36]. Regarding ceftazidime, a 3rd generation cephalosporin and not recommended for veterinary use, the interpretation according to CLSI guidelines revealed 54% susceptible, whereas the interpretation according to EUCAST revealed only 14% susceptible isolates (Table 4) [15-17]. Further comparison of the two guidelines, it can be seen that data to certain antibiotics are missing (Table 2). The CLSI did not publish values for meropenem, and the EUCAST did not comment on cefoxitin [15, 17]. Cefoxitin is a 2nd generation cephalosporin, and is admitted as well as frequently used for the application in dogs and companion animals [19]. Out of the investigated 97 Enterobacteriaceae isolates in the present study, only 16% revealed to be resistant [36]. Interpreting this low percentage it may be assumed that the antimicrobial resistance is of other origin than previous antibiotic treatment in the namely dog. Although comprehensible that the two committees regarding antimicrobial resistance published different guidelines, this discrepancy just seems too large. Especially considering that the MIC guidelines greatly influence the recommendation for drug application in human or animal patients worldwide.

Table 4: Number of the isolates regarding the reaction in presence of ceftazidime

Number of isolates (n)	CLSI [15]	EUCAST [17]
susceptible	53	3
intermediate	10	39
resistant	35	45
total assigned isolates	98	98
unassigned	3	3

Currently, publications include more and more molecular results, and even data regarding whole genome analysis and information about coding sequences (CDSs) regarding antimicrobial resistance [12, 36, 39, 43, 73]. Therefore and as previously discussed the presence or absence of these CDSs should be taken into account or even rated equally as

certain MIC values. It should be considered that silent genes may be quickly activated in presence of an antimicrobial substance. Following this, also the non-Enterobacteriaceae organisms that are currently not considered in the CLSI or EUCAST guidelines for ESBL detection, would also be covered [12]. In the present study, A*eromonas caviae*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Pseudomonas fulva* were amongst the resistant isolates (4%) [36]. Only very limited MIC-interpretation regarding the species *Pseudomonas aeruginosa* is currently available according to published guidelines [15-17]. The carbapenem-resistance is a common phenomenon published for *Pseudomonas* species [71]. This could be confirmed in the present study, as the two *Pseudomonas* isolates revealed a MIC of 1 µg/ml, whereas all other isolates were completely susceptible towards ertapenem [36].

A predominant opinion is that ESBL-producing *E. coli* of animal origin are a major source of human infections. However, different studies revealed that about half of all hospital associated ESBL-producing isolates revealed the MLST sequencetype 131 [73-74]. These MLST ST131 ESBL-producing *E. coli* were not detected amongst the isolates of the present study [36]. We can therefore clearly support a published statement that the above-named opinion is oversimplified and neglected the complex transmission pathways [43]. Concluding the results of our study, we could bring some more insight into the broad research field of veterinary antimicrobial resistance. We did not find any so called human related MLST sequencetypes in this study, despite there can be a higher risk for special risk-groups, for example farmers, veterinarians or in this case dog handlers. A risk-evaluation can only be conducted in an one health approach, further research is mandatory.

V. Zusammenfassung

Die Zunahme antibiotikaresistenter Bakterien stellt aktuell ein großes Problem im Bereich der klinischen Mikrobiologie dar. Komplikationen bei der Behandlung von Infektionskrankheiten, die auf die bakterielle Resistenz zurückzuführen sind, reichen von verlängerten Behandlungszeiten bis hin zu Todesfällen. Beta-Laktam-Antibiotika, wie Penicilline und Cephalosporine werden in der Veterinärmedizin häufig, Carbapeneme nur als Reserveantibiotika angewendet. Enterobakterien können gegen diese Antibiotika schnell und vielfältige Resistenzen entwickeln. Bereits 1979 wurden erste Beta-Laktamase-bildende und mit erweitertem Wirkungsspektrum „extended-spectrum“ Beta-Laktamase-bildende (ESBL) Enterobakterien beschrieben. Seit den 1990er Jahren ist bekannt, dass bakterielle Resistenzen vermehrt bei antibiotikatherapierten Hunden auftreten. Heute weiß man, dass Menschen, eine Vielzahl an Säugetierarten und auch Vögel asymptomatisch mit ESBL-bildenden Enterobakterien besiedelt sein können.
In der vorliegenden Arbeit zur Charakterisierung von ESBL-bildenden *Escherichia coli* in Diensthunden der Bundeswehr wurden antibiotikaresistente Bakterien aus Hundekot isoliert. Die beprobten Tiere lebten zu diesem Zeitpunkt zeitweise mit anderen Diensthunden in einer Hundewache und teilweise mit weiteren Begleithunden in Privathaushalten zusammen. Die meisten Isolate wurden im Rahmen einer Langzeitstudie über elf Monate von den Diensthunden gesammelt. Letztere waren mindestens ein Jahr vor der ersten Probenahme bis zum Abschluss der letzten Probenahme nach elf Monaten nicht antibiotisch behandelt worden. Für die vorliegende Studie konnten weitere Bakterienisolate von streunenden Hunden aus den Einsatzgebieten der Bundeswehr gewonnen werden. Insgesamt wurden 101 Bakterienisolate aus Deutschland, der Republik Kosovo, Afghanistan, Kroatien und der Ukraine untersucht. Nach der klassischen mikrobiologischen Identifizierung von verdächtigen Bakterien wurde zunächst eine *in vitro* Empfindlichkeitstestung mit dem Bouillon-Mikrodilutionsverfahren durchgeführt. Die getesteten Substanzen stammten aus der Gruppe der Cephalosporine, hier Cefoxitin, Cefotaxim, Ceftazidim und Cefepim. Die drei letztgenannten wurden auch in Kombination mit Clavulansäure getestet. Die Testung erfolgte zudem mit den Carbapenem-Antibiotika Ertapenem und Meropenem. Die ermittelte minimale Hemmkonzentration (MHK, minimal inhibitory concentration, MIC) zeigte, dass es

verschiedene Resistenzmuster unter den untersuchten Isolaten gab. Keines der 101 untersuchten Isolate war gegen eines der Carbapenem-Antibiotika resistent.

Weiterhin wurden Vollgenomsequenzierung und -analyse der Isolate durchgeführt, um die *in vitro* Daten zu ergänzen und molekularbiologische Daten zu generieren. In Bezug auf Antibiotikaresistenz konnten 23 verschiedene Gene identifiziert werden, die spezifisch zu den identifizierten Bakterienspezies zugeordnet werden konnten und die eine Antibiotikaresistenz der Bakterien molekularbiologisch bewiesen. Eine molekulare Verwandtschaftsanalyse wurde anhand der ermittelten kanonischen single nucleotide polymorphisms (SNPs) vorgenommen. Die Ergebnisse zeigten für wenige Isolate eine klonale Verwandtschaft. Dies bewies eine direkte Tier-zu-Tier Übertragung von Isolaten aus der Langzeitstudie. Jedoch konnten diese Bakterienklone nur innerhalb von maximal sieben aufeinanderfolgenden Tagen isoliert werden, eine stabile Ausscheidung spezifischer Gene über einen längeren Zeitraum konnte nicht belegt werden. Schließlich wurde die Methode multi locus sequence typing (MLST) von den 85 antibiotikaresistenten *E. coli* Isolaten durchgeführt. Diese erbrachte 31 unterschiedliche Sequenztypen (ST) von welchen die Sequenztypen ST744 (n=9), ST10 (n=8) und ST648 (n=6) am häufigsten vertreten waren. Der aus der Humanmedizin weltweit beschriebene Krankenhaus-Problemkeim mit der Beta-Lactamase CTX-M und einem MLST Sequenztyp ST131 wurde in der vorliegenden Studie nicht identifiziert. Die epidemiologische Interpretation ergab keine weitere Korrelation der Isolate aus der Langzeitstudie untereinander. Auch wurde keine signifikante Verwandtschaft von Isolaten mit unterschiedlicher geografischer Herkunft festgestellt.

Die Ergebnisse der vorliegenden Arbeit bewiesen die hohe Prävalenz von ESBL-produzierenden Bakterien bei (gesunden) Hunden, die keiner Antibiotikatherapie unterzogen worden waren. Es wurde gezeigt, dass einzelne Isolate zwischen den Tieren einer Gruppe übertragen wurden. Diese Bakterienklone wurden jedoch nach kurzer Zeit im Tier wieder eliminiert. Im Vergleich zu publizierten Daten gab es auch in dieser Studie keinen Anhaltspunkt, dass es Hunde-typische Isolate mit spezifischem Resistenzmuster gibt. Die Daten aus der Vollgenomsequenzierung konnten im Rahmen dieser Arbeit öffentlich publiziert werden. Für künftige Studien stehen diese daher zur Verfügung und ergänzen mögliche epidemiologische Fragen im spannenden Forschungsfeld über antibiotikaresistente Bakterien.

VI. Summary

Antimicrobial resistance is a growing concern in clinical microbiology today. Regarding the unsuccessful treatment of bacterial infections in human or animal patients, consequences reach from extended treatment times through complications and may end with a death of an individual patient due to untreatable bacterial infection. The beta-lactam antibiotics, namely penicillins, the cephalosporins (rarely the carbapenems) are highly used in veterinary medicine. Enterobacteriaceae easily develop a broad variety of antimicrobial resistance. Beta-lactamase producing bacteria, or even more powerful „extended-spectrum“ beta-lactamase producing Enterobacteriaceae (ESBL) have been described since 1979. Since the 1990s bacterial resistance against beta-lactam antibiotics was found in dogs. Currently the asymptomatic colonization with extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae has been described for humans, various mammal species, and birds.

In the present study about the characterization of ESBL-producing *Escherichia coli* in military working dogs in the German Armed Forces antimicrobial resistant bacteria were recovered from dog feces. During the study time, the dogs lived together in a group of working dogs, but also stayed with companion dogs at the home of their family. Most of the isolates were obtained from the military working dogs within a longitudinal collection over eleven months. The dogs had not been treated with antibiotics during the past year until the beginning of the study period. More study isolates were recovered from stray dogs originating from the theatre of operations of the German Armed Forces. In all 101 bacterial isolates were investigated in the present study originating from Germany, Republic of Kosovo, Afghanistan, Croatia and Ukraine.

To characterize the bacterial isolates, the *in vitro* antimicrobial susceptibility testing was carried out using the extended-spectrum cephalosporins cefoxitin, cefotaxime, ceftazidime, and cefepime, with the last three listed also tested in combination with clavulanic acid. As well, susceptibility testing was done using the carbapenem antibiotics ertapenem and meropenem. The determined minimal inhibitory concentration (MIC) values revealed divers resistance patterns against single or all investigated beta-lactam antibiotics, with none of the 101 isolates resistant against the two tested carbapenem antibiotics.

Furthermore, whole genome sequence analysis was carried out to support the *in vitro* data and revealed an insight into molecular data. Regarding antimicrobial resistance 23 different but species-specific coding DNA sequences (CDS) were identified proving antimicrobial resistance on a molecular basis. A phylogenetic analysis was carried out using canonical single nucleotide polymorphisms (SNPs). The results revealed clonal bacterial isolates originating from different dogs, suggesting transmission between dogs from the same community. These clonal isolates however were not detected over a period longer than seven days. Finally performing multi locus sequence typing (MLST) out of the 85 resistant *E. coli* isolates identified 31 different sequence types (ST). The most frequent ST were ST744 (n=9), ST10 (n=8), and ST648 (n=6), respectively. Amongst these, the world-wide human hospital-associated CTX-M beta-lactamase producing ST131 was not detected. Further epidemiologic interpretation did not support a correlation among the longitudinal isolates. There was no molecular proof of relationship between dog-isolates of different geographic origin.

The data of the present thesis proof a high prevalence of ESBL-producing bacteria in healthy dogs, independent to prior treatment with antibiotics. It could be shown that single isolates were transmitted between individuals of the same community. These isolates however were as well eliminated after a short time. Most of the characterized bacteria revealed few characteristics signing them host-specific for dogs at this point. Within the present study, the whole genome analysis data were publicly published and are available to contribute for future epidemiologic questions regarding the exciting research field of antimicrobial resistant bacteria.

VII. References

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