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Multiresistant extended-spectrum β-lactamase-producing *Enterobacteriaceae* from humans, companion animals and horses in central Hesse, Germany

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Abstract

Background: Multiresistant Gram-negative bacteria producing extended-spectrum β-lactamases (ESBLs) are an emerging problem in human and veterinary medicine. This study focused on comparative molecular characterization of β-lactamase and ESBL-producing *Enterobacteriaceae* isolates from central Hesse in Germany. Isolates originated from humans, companion animals (dogs and cats) and horses.

Results: In this study 153 (83.6%) of the human isolates (n = 183) and 163 (91.6%) of the animal isolates (n = 178) were confirmed as ESBL producers by PCR and subsequent sequencing of the PCR amplicons. Predominant ESBL subtypes in human and animal samples were CTX-M-15 (49.3%) and CTX-M-1 (25.8%) respectively. Subtype $bla_{CTX-M-2}$ was found almost exclusively in equine and was absent from human isolates. The carbapenemase OXA-48 was detected in 19 ertapenem-resistant companion animal isolates in this study. The Plasmid-encoded quinolone resistance (PMQR) gene *aac('6)-Ib-cr* was the most frequently detected antibiotic-resistance gene present in 27.9% of the human and 36.9% of the animal ciprofloxacin-resistant isolates. Combinations of two or up to six different resistance genes (penicillinases, ESBLs and PMQR) were detected in 70% of all isolates investigated. The most frequent species in this study was *Escherichia coli* (74%), followed by *Klebsiella pneumoniae* (17.5%), and *Enterobacter cloacae* (4.2%). Investigation of *Escherichia coli* phylogenetic groups revealed underrepresentation of group B2 within the animal isolates.

Conclusions: Isolates from human, companion animals and horses shared several characteristics regarding presence of ESBL, PMQR and combination of different resistance genes. The results indicate active transmission and dissemination of multi-resistant *Enterobacteriaceae* among human and animal populations.

Keywords: ESBL, *Enterobacteriaceae*, Comparison, Human isolates, Animal isolates, One Health concept

Background

Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* are isolated with increasing frequency from human and animal samples. In particular, the species *Escherichia (E.) coli* plays an important role as a source of the corresponding resistance genes [1]. ESBLs genes are commonly plasmid-encoded and can easily be transmitted by conjugation to other bacteria, even across species barriers. In addition, various resistance genes are located in or close to mobile genetic elements such as insertion sequences and transposons [2]. Lateral gene transfer and continuous DNA recombination make it extremely difficult to track transmission pathways of ESBL genes in bacterial populations. The transmission of ESBL-producing pathogens or ESBL genes between companion animals/livestock and owner/caretaker/consumer is currently a subject of intense and controversial discussion. Evidence has been presented for zoonotic

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spread [3,4]. Several studies have addressed the risk of infection that could arise from keeping animals [5-7]. A recent study highlighted that the risk factors for ESBL carriage were travel to Greece or Africa within the last 12 months and keeping of pet animals, but not antibiotic consumption or recent hospitalization [8]. Until now, it is still difficult to estimate the amount of exchange and even more difficult to define the risk for human and animal health and also food safety.

The focus of this study was a comparative investigation of 361 ESBL-producing *Enterobacteriaceae* isolates obtained from animals and human patients presenting at a veterinary clinic and a hospital, respectively, both serving a similar catchment area. The prevalence of β-lactamase-, particularly ESBL-producing bacteria in companion animal, horse and human isolates of clinical origin was examined. Resistance genes (β-lactamase and plasmid-mediated quinolone resistance (PMQR)) and *E. coli* phylogenetic groups were investigated using molecular methods.

This work sheds light on shared populations of ESBL-producing *Enterobacteriaceae* in symptomatic companion animals, horses and humans in the geographical region of middle Hesse, Germany.

Methods
Bacterial isolates
A collection of 513 *Enterobacteriaceae* isolates resistant to one or more third-generation cephalosporins (200 human isolates and 313 animal isolates) was examined. Identification was done using in-house biochemical tests. In case of ambiguous results confirmation was done using API 20E. Prior to further analysis, all isolates were grown on MacConkey agar supplemented with cefotaxime (2 mg/l) to promote selection of β-lactamase producers and ensure selection of *Enterobacteriaceae*. Afterwards isolates were tested for possible ESBL production by double disc synergy test (DDST) according to EUCAST guidelines for resistance mechanisms [9]. After performance of the DDST 183 human and 178 animal isolates were categorized as possible ESBL-producers and forwarded for a more detailed investigation. Isolates with a negative DDST result were not included in the study.

DDST-positive isolates from human clinical samples (n = 183) were taken from the strain collection of the Institute of Medical Microbiology, Giessen. Some of the isolates originate from routine screening for colonization with ESBL-producing *Enterobacteriaceae* at the University hospital Giessen. Additional isolates were collected from samples of clinically ill patients e.g. blood culture or urine samples in the same facility. All isolates were collected between 2009 and 2010. Approximately two-thirds of the human isolates originated from inpatients (n = 128) and one third were isolates from outpatients wards (n = 55).

DDST-positive isolates from animals (n = 178) were obtained during a survey for aerobic Gram-negative bacteria growing on MacConkey agar supplemented with cefotaxime (2 mg/l) among animal patients at the veterinary clinics of the Justus Liebig University (JLU) Giessen. All samples were clinically relevant infection related isolates. The isolates originated from horses (n = 100), dogs (n = 67) and cats (n = 11) and were collected between 2009 and 2011.

The study was approved by the ethics committee of medical faculty of the Justus Liebig University of Giessen and deemed exempt from informed consent.

Antibiotic susceptibility
Antibiotic susceptibility testing was done using the VITEK®2 compact system with AST N117 cards (Biomérieux) and Etest® stripes (Liofilchem®) containing ertapenem, cefepime, chloramphenicol and nalidixic acid. Results were evaluated according to CLSI guidelines for human pathogens (CLSI, 2012).

β-lactamase identification
For antibiotic resistance genes screening by PCR and identification by sequencing were performed as described previously [10]. Specific oligonucleotide primers for bla_{TEM}, bla_{SHV} [11], bla_{CTX-M} genes [12] and bla_{OXA-1} [13] were used. The MAST carbapenemase detection set (MAST group, UK) was applied on carbapenem-resistant isolates. Furthermore, primers for bla_{OXA-48} were used for carbapenem-resistant isolates [11]. With the exception of bla_{OXA-1}-positive strains, amplicons of PCR-positive strains were sequenced to identify the encoded ESBL allele in detail. Sequencing was performed using the automated sequencer ABI Prism® 3100 (Life technologies, USA). The blastn algorithm of NCBI (http://www.ncbi.nlm.nih.gov/blast/) was used for database searches to identify the resistance gene allele. The primers and the sequences they were derived from are presented in Table 1. Positive control strains from the Institute of Microbiology, Giessen, Germany, were *Klebsiella (K.) pneumoniae* H59 for bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA-1} and *aac(6')-Ib*, *Klebsiella pneumoniae* 714 for bla_{OXA-48}. For the *qnr*-genes, strains from the Institute of Hygiene and Infectious Diseases of Animals, Giessen, Germany (V148 for *qnrA*, V167 for *qnrB*, V60 for *qnrD* and V61 for *qnrS*), were used as positive controls after sequencing and comparison with known sequences using DNASTAR software (DNASTAR Inc, Madison, USA) and the NCBI blastn algorithm.

Detection of Plasmid-encoded quinolone resistance (PMQR)
Detection for PMQR by PCR was performed on all isolates with a minimum inhibitory concentration (MIC) ≥

Table 1 PCR primers used to detect β-lactamase genes, PMQR genes and _E. coli_ phylogenetic groups

Primer	Sequence (5' - 3')	Target	Size of product (bp)	Reference
TEM_f	ATGAGTATTCAACATTTCCG	bla_{TEM}	851	[11]
TEM_r	TTAATCAGTGAGGCACCTAT			
SHV_f	GCAAAACGCCGGGTTATTC	bla_{SHV}	940	[11]
SHV_r	GGTTAGCGTTGCCAGTGCT			
CTX-M_f	TCTTCCAGAATAAGGAATCCC	bla_{CTX-M}	909	[12]
CTX-M_r	CCGTTTCCGCTATTACAAAC			
OXA-1_f	AGCAGCGCCAGTGCATCA	bla_{OXA-1}	700	[13]
OXA-1_r	ATTCGACCCCAAGTTTCC			
OXA-48_f	AAATCACAGGGCGTAGTTGTG	bla_{OXA-48}	555	[11]
OXA-48_r	GACCCACCAGCCAATCTTAG			
qnrA_f	AGAGGATTTCTCACGCCAGG	qnrA	619	[14]
qnrA_r	GCAGCACTATKACTCCCAAGG			
qnrB_f	GGMATHGAAATTCGCCACTG	qnrB	264	[16]
qnrB_r	TTTGCYGYYCGCCAGTCGAA			
qnrC_f	GGGTTGTACATTTATTGAATC	qnrC	447	[17]
qnrC_r	TCCACTTTACGAGGTTCT			
qnrD_f	CGAGATCAATTTACGGGGAATA	qnrD	582	[18]
qnrD_r	AACAAGCTGAAGCGCCTG			
qnrS_f	GCAAGTTCATTGAACAGGCT	qnrS	428	[16]
qnrS_r	TCTAAACCGTCGAGTTCGGCG			
qepA_f	CTGCAGGTACTGCGTCATG	qepA	403	[15]
qepA_r	CGTGTTGCTGGAGTTCTTC			
aac_f	TTGCGATGCTCTATGAGTGGCTA	aac(6')-Ib	482	[19]
aac_r	CTCGAATGCCTGGCGTGTTT			
chuA.1	GACGAACCAACGGTCAGGAT	chuA	279	[21]
chuA.2	TGCCGCCAGTACCAAAGACA			
YjaA.1	TGAAGTGTCAGGAGACGCTG	yajA	211	[21]
YjaA.2	ATGGAGAATGCGTTCCTCAAC			
TspE4C2.1	GAGTAATGTCGGGGCATTCA	TspE4C2	152	[21]
TspE4C2.2	CGCGCCAACAAAGTATTACG			

1 μg/ml for ciprofloxacin (Human isolates: n = 140, animal isolates: n = 122). Oligonucleotide primers for detection of _qnrA, qnrB, qnrC, qnrD, qnrS, qepA and aac(6')-Ib_ were used [14-19]. Identification of the _aac(6')-Ib-cr_ variant was performed according to Jones et al. 2008 [20].

Phylogenetic groups of ESBL-producers

Distribution of _E. coli_ phylogenetic groups was analysed by PCR targeting genes _chuA, yjaA_ and the DNA fragment TSPE4.C2 to assign the strains to the groups A, B1, B2 and D according to Clermont et al. 2000 [21].

Analysis of resistance genes patterns

Information regarding the source of isolates, species and the resistance genes detected were assembled into an Excel file. The data was subsequently analysed using GENE-E [22] which enables the clustering of data with matrix visualization and analysis to support visual data exploration.

Results

Overview of bacterial species

The majority of the isolates studied were _E. coli_ (74%), followed by _K. pneumoniae_ (17.5%) and _Enterobacter cloacae_ (4.2%). Other species detected were _Klebsiella oxytoca, Enterobacter intermedius, Citrobacter freundii, Providencia stuartii, Morganella morganii_ and _Proteus mirabilis_. The percentages of _E. coli_ and _K. pneumoniae_ isolates among the human and animal isolates were very similar.

Resistance rates

All isolates (n = 361) were categorised as multidrug resistant (MDR) according to the international expert proposal for interim standard definitions for acquired resistance promoted by the ECDC [23]. Both human (n = 183) and animal isolates (n = 178) revealed resistance against ampicillin and trimethoprim/sulfmethoxazole. Slight differences could be observed concerning the tested third generation cephalosporins ceftazidime, cefotaxime and cefepime. Of the human isolates 52.5% (n = 96) showed resistance against ceftazidime, 78.7% (n = 144) resistance against cefotaxime and 98.4% (n = 180) resistance against cefepime. Of the animal isolates 75.8% (n = 135) displayed resistance against ceftazidime and 99.4% (n = 177) to cefotaxime and cefepime. Of the human isolates 2.2% (n = 4), and 1.1% (n = 2) of the animal isolates revealed resistance against imipenem. Resistance against the other tested carbapenem (ertapenem) was detected in 24.5% (n = 45) of human isolates and in 19.7% (n = 35) of animal isolates. The aminoglycosides gentamicin and amikacin differed in their results.

Only 5.1% (n = 9) of animal isolates exhibited resistance against amikacin, as compared to 14.8% (n = 27) of the human isolates. The resistance rates against gentamicin were higher in both cases with 35% (n = 64) in human isolates and 62.4% (n = 111) in animal isolates. High resistance rates in both groups were also revealed for the fluoroquinolone ciprofloxacin (76.5% (n = 140) of the human isolates and 68.5% (n = 122) of the animal isolates).

Distribution of β-lactamases

Almost all isolates carried at least one beta-lactamase (*bla*)-gene (Table 2). In all, 91.6% of the animal isolates and 83.6% of the human isolates were confirmed as ESBL producers by PCR and sequencing. The remaining isolates (7.5%) were negative for the presence of bla_{TEM}, bla_{SHV}, bla_{OXA-1}, bla_{OXA-48} and bla_{CTX-M}.

CTX-M

Subtypes of the ESBL CTX-M were by far the most frequently encoded ESBLs in both groups (Table 2 and

Table 2 Distribution of β-lactamase genes in *Enterobacteriaceae* isolates (n = 361) of human and animal origin

Source	Human		Animal			
	Outpatients	Inpatients	Dogs	Cats	Horses	Total no. of isolates
No. of isolates[a]	55	128	67	11	100	361
Penicillinases[b]						
bla_{TEM-1}	58.2 (32)	41.4 (53)	62.7 (42)	45.5 (5)	67 (67)	55.1 (199)
$bla_{TEM-190}$			1.5 (1)			0.3 (1)
bla_{SHV-1}		2.3 (3)	16.4 (11)		2 (2)	4.4 (16)
bla_{SHV-11}		1.6 (2)				0.6 (2)
bla_{OXA-1}	23.6 (13)	32.8 (42)	59.7 (40)	45.5 (5)	29 (29)	35.7 (129)
ESBL[b]						
bla_{TEM-52}		2.3 (3)				0.8 (3)
bla_{SHV-2}			1.5 (1)	9.1 (1)		0.6 (2)
bla_{SHV-5}		0.8 (1)				0.3 (1)
bla_{SHV-28}			3(2)		1(1)	0.8 (3)
$bla_{CTX-M-1}$	32.7 (18)	45.3 (24)	16.4 (11)	27.3 (3)	37 (37)	25.8 (93)
$bla_{CTX-M-2}$				9.1 (1)	15 (15)	4.4 (16)
$bla_{CTX-M-9}$			3 (2)	9.1 (1)	1 (1)	1.1 (4)
$bla_{CTX-M-14}$			1.5 (1)			0.3 (1)
$bla_{CTX-M-15}$	54.5 (30)	51.6 (66)	59.7 (40)	36.4 (4)	38 (38)	49.3 (178)
$bla_{CTX-M-32}$	1.8 (1)	0.8 (1)				0.6 (2)
$bla_{CTX-M-38}$		0.8 (1)				0.3 (1)
$bla_{CTX-M-79}$	1.8 (1)	2.3 (3)			1(1)	1.4 (5)
$bla_{CTX-M-97}$	1.8 (1)				3(3)	1.1 (4)
$bla_{CTX-M-117}$		0.8 (1)				0.3 (1)
Carbapenemases[b]						
bla_{OXA-48}			23.9 (16)	18.2 (2)	1(1)	5.3 (19)

[a]numerous isolates encoded for more than one β-lactamase gene.
[b]% of β-lactamase genes (no. of isolates).

Figure 1). Among the human isolates 23% encoded CTX-M-1 and 52.5% CTX-M-15. Some human isolates carried $bla_{CTX-M-79}$, $bla_{CTX-M-32}$, $bla_{CTX-M-97}$, $bla_{CTX-M-38}$ or $bla_{CTX-M-117}$. The $bla_{CTX-M-1}$ gene was found in 32.7% of human outpatient isolates and in 45.3% of inpatient isolates, while prevalence rates of $bla_{CTX-M-15}$ were high and similar in both groups (outpatients 54.5%, inpatients 51.6%). Animal isolates revealed similar results as 28.7% of these isolates carried $bla_{CTX-M-1}$ and 46.1% $bla_{CTX-M-15}$. Other detected bla_{CTX-M} genes were $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, $bla_{CTX-M-97}$, $bla_{CTX-M-79}$, and $bla_{CTX-M-14}$. The presence of $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, and $bla_{CTX-M-14}$ was restricted to animal isolates, while $bla_{CTX-M-32}$, $bla_{CTX-M-38}$, $bla_{CTX-M-117}$ were found only among the human isolates. $Bla_{CTX-M-1}$ was the dominant resistance subtype in horses (37%) but it was less frequently isolated in dogs (16.4%). In contrast, $bla_{CTX-M-15}$ occurred more often in dog isolates (59.7%) than in isolates from horses (38%) or cats (36.4%). In addition, $bla_{CTX-M-15}$ was also the dominant CTX-M subtype among *K. pneumoniae* isolates whereas among the *E. coli* isolates both subtypes were present to the same degree. Altogether, $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$ were the prominent β-lactamase genes present in 25.8% and 49.3% of all investigated isolates.

TEM

Non-ESBL gene bla_{TEM-1} was the most frequently identified β-lactamase gene among all investigated isolates. It was detected in 46.4% of the human isolates and in 64% of all animal isolates. It was present in 199 of 361 isolates (55%). The highest detection rates were observed in outpatients (58.2%), inpatients (41.4%), dog isolates (62.7%) and horse isolates (67%). TEM-52 was the only TEM-type ESBL that occurred in this study. It was identified in three isolates from human inpatients. Results are shown in Table 2.

SHV

Prevalence of bla_{SHV} was 17.2% in human and 52.9% in animal *K. pneumoniae*. Bla_{SHV-1} was identified in 10.3% of the human and in 29.4% of the animal *K. pneumoniae* isolates. Several ESBL variants could be identified in addition (Table 2). Most of the latter isolates originated from dogs. Detected SHV-type ESBL genes were bla_{SHV-2}, bla_{SHV-5}, bla_{SHV-11} and bla_{SHV-28}. Bla_{SHV-5} and bla_{SHV-11} were restricted to isolates from human inpatients. ESBL genes bla_{SHV-2} and bla_{SHV-28} were detected among the animal isolates only.

OXA-1

The presence of the penicillinase gene bla_{OXA-1} could be demonstrated in 30.1% of the human isolates. Among the animal isolates bla_{OXA-1} was identified in 41.6% of

all strains. Detection rates were highest in isolates from dogs (59.7%), followed by cat (45.5%), outpatient (32.8%) and horse (29%) isolates. However, bla_{OXA-1} was also found in inpatient isolates (23.6%) (Table 2).

OXA-48

All carbapenem resistant isolates (human isolates: 45, animal isolates: 40) were tested for presence of the carbapenemase OXA-48. None of the tested human isolates harboured bla_{OXA-48}. In contrast, 19 (5.3%) of the animal isolates harboured this gene. The isolates harbouring bla_{OXA-48} originated from dogs (16), cats (2) and a horse (Table 2).

PMQR

Distribution and presence of PMQR genes are illustrated in Table 3. The most frequently detected PMQR gene in ciprofloxacin-resistant isolates (n = 262) was *aac('6)-Ib-cr*. Prevalence was 27.9% (n = 39) in the human isolates, 36.9% (n = 45) in the animal isolates and 32.1% (n = 84) in all investigated isolates. None of the isolates carried *qnrC* or *qepA*. Among the human isolates *qnrB* was found in three and *qnrS* in five isolates. Two more *qnr* variants were detected among the animal isolates. Gene *qnrA* was carried by three, *qnrB* by seven, *qnrD* by eight and *qnrS* by five animal isolates. Most *qnr* type genes (*qnrA, qnrB, qnrD, qnrS*) were detected in dog isolates. Inpatient isolates carried PMQR genes at higher rates (37.8%) than isolates from outpatients (23.8%). A similar observation could be made among the animal isolates. Highest identification rates (49.5%) were observed in isolates originating from animals tested during stay in the small animal clinic. In summary, most PMQR genes were present in dog isolates (65.1%). Overall, PMQR genes could be demonstrated in 43.9% (n = 115) of all investigated isolates.

Analysis of resistance gene patterns

Preliminary detection and visualization of clusters and/ or resistance patterns in context to isolate source and bacterial species was performed using Figure 1 which was generated by means of the GENE-E program. Data was then confirmed by analysis of absolute and relative numbers/ percentages as displayed in Table 4. Combinations of various plasmid encoded resistance genes (ESBL and non-ESBL β-lactamases and PMQR) were observed in the majority of isolates (70.1%) included in this study (Table 4 and Figure 1). Most frequently detected (26.3%) was the combination of a TEM type penicillinase with a CTX-M type ESBL. Also regularly observed was a penicillinase (TEM or OXA-1) in combination with CTX-M and plasmid-mediated quinolone resistance (PMQR) genes. These combinations were predominantly found among *E. coli* isolates. Other isolates encoded for
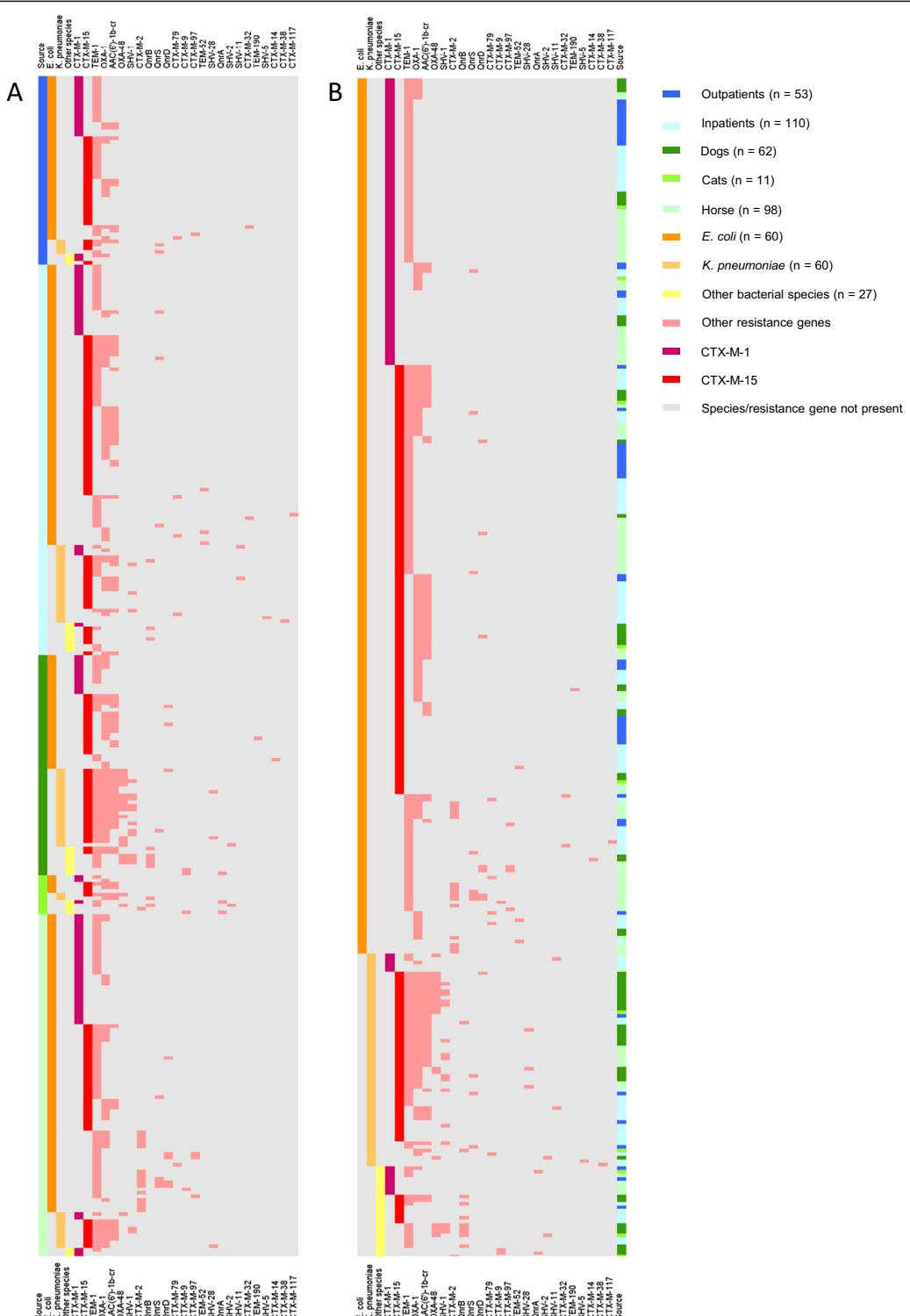
Figure 1 Heat maps generated from identified resistance genes and bacterial species among human and animal isolates. Identified resistance genes and bacterial species are listed according to frequency (except for CTX-M-1 and CTX-M-15) on the right and left side of the figures. Source = origin of isolates (outpatients, inpatients, dogs, cats, horses). Not included are isolates without detectable resistance gene (n = 28). The term "Other species" includes *Enterobacter cloacae* (n = 12), *Klebsiella oxytoca* (n = 9), *Enterobacter intermedius* (n = 2), *Enterobacter gergoviae* (n = 1), *Citrobacter freundii* (n = 1) and *Proteus mirabilis* (n = 1). **A** is focussing on the origin of isolates whereas the **B** emphasizes the involved bacterial species.

Table 3 Distribution of PMQR genes in ciprofloxacin-resistant isolates (n = 262) of human and animal origin

Source	No. of isolates[b]	% of PMQR genes (no. of isolates)						
		qnrA	qnrB	qnrC	qnrD	qnrS	qepA	aac(6')-Ib-cr
Human								
Outpatients	42					4.8 (2)		19 (8)
Inpatients	98		3.1 (3)			3.1 (3)		31.6 (31)
Total	140		2.1 (3)			3.6 (5)		27.9 (39)
Animal								
Dogs	63	1.6 (1)	7.9 (5)		4.8 (3)	1.6 (1)		49.2 (31)
Cats	10	20 (2)	20 (2)					40 (4)
Horses	49				10.2 (5)	8.2 (4)		20.4 (10)
Total	122	2.5 (3)	5.7 (7)		6.6 (8)	4.1 (5)		36.9 (45)
Total no. of isolates	262	1.1 (3)	3.8 (10)		3.1 (8)	3.8 (10)		32.1 (84)

[b]some isolates encoded for more than one PMQR gene.

several combinations of a TEM penicillinase, CTX-M enzymes, the penicillinase bla_{OXA-1}, the penicillinase bla_{SHV} and PMQR genes. The carbapenemase OXA-48 was mainly associated with numerous resistance genes such as a TEM penicillinase, CTX-M and PMQR. In addition, it was exclusively identified in *K. pneumoniae* and *Enterobacter cloacae* isolates from animals. Another frequently observed combination was that of $bla_{CTX-M-15}$ and *aac('6)-Ib-cr*. Other combinations were detected only once or to a much lesser extent than those mentioned above. Combination of more than two resistance genes could be observed more often among human *E. coli* isolates and especially among *K. pneumoniae* animal isolates (Figure 1).

Phylogenetic grouping of β-lactamase-producing E. coli
Phylogenetic group typing was applied to 141 human and 126 animal ESBL-producing *E. coli* isolates (Table 5 and Figure 2). Both groups differed from each other in the frequencies of known phylogenetic groups. In general, the human isolates were almost evenly distributed among the phylogenetic groups A (30.5%), B1 (27.7%) and B2 (26.2%) and to a lesser extent with group D (15.6%). In contrast, most of the analysed animal isolates belonged to group A (43.7%) and a considerably smaller number of isolates to B1 (35.7%). Approximately 17.5% of all animal isolates could be assigned to group D, whereas only 2.4% were classified as group B2.

Discussion
The global spread of multi-resistant ESBL-producing *Enterobacteriaceae* can in part be explained by mobility of the resistance bearing genetic elements e.g. plasmids, transposons and insertion sequence elements. This fact is a major concern in terms of epidemiology and infection control. Transmission pathways between humans and animals are currently a subject of active discussion [8,24,25]. This study was designed to investigate the distribution of β-lactamases, particularly ESBL, and PMQR genes among isolates from human, companion animal and horse samples in a defined geographical area of Germany. The key question was whether similar resistance characteristics are currently present among both groups. Indeed, our studies here demonstrate that isolates from human and animal samples share numerous characteristics.

Antimicrobial susceptibility testing revealed common and disturbing patterns. High resistance rates for all β-lactams and other antibiotic classes were observed. In particular animal isolates harbouring resistance to the last-resort antibiotics ertapenem and impenem were detected. The results for ertapenem were particularly alarming, with 16.3% resistance among animal isolates and 24% among the human isolates. In Germany, the use of carbapenems in livestock animals is prohibited and its use for treating companion animals is strongly restricted. Nevertheless, almost all ertapenem resistant strains among the animal isolates (95.6%) originated from dogs and cats. This would suggest human to animal transmission of resistant strains by their owners. Alternatively, the use of carbapenems for treatment of companion animals cannot be excluded even though these pharmaceuticals are expensive and their use is uncommon even in pet animal practice. Carbapenemase activity in bacteria from companion animals and livestock has been already identified recently [26-28]. To investigate this phenomenon in more detail, all carbapenem resistant isolates from this study were tested for the presence of bla_{OXA-48}. Surprisingly, a substantial difference could be observed regarding the molecular mechanisms underlying this resistance phenotype. None of the 45 tested human isolates was positive for bla_{OXA-48}, though 26 (57.8%) were identified with a possible combination of an AmpC enzyme and porin deficiency. This mechanism has been observed previously in AmpC-producing *K. pneumoniae* by Shi et al. They stated

Table 4 Observed combinations of β-lactamase and PMQR genes according to bacterial species

Combination of resistance genes[a]	Outpatients					Inpatients					Dogs					Cats					Horses				
	% of bacterial species (no. of isolates)																								
	EC	KP	KO	EN	OS[b]	EC	KP	KO	EN	OS[b]	EC	KP	KO	EN	OS[b]	EC	KP	KO	EN	OS[b]	EC	KP	KO	EN	OS[b]
TEM$_{Pen}$/CTX-M	50 (24)					26.9 (25)	12 (3)				17.1 (6)					20 (1)					40.7 (35)				25 (1)
TEM$_{Pen}$/CTX-M/PMQR		25 (1)				1.2 (1)			25 (1)		2.9 (1)					20 (1)			33.3 (1)		8.1 (7)				
TEM$_{Pen}$/CTX-M/OXA$_{Pen}$	2.1 (1)		33.3 (1)			2.2 (2)					14.3 (5)										12.8 (11)	10 (1)			
TEM$_{Pen}$/CTX-M/OXA$_{Pen}$/PMQR	4.2 (2)	25 (1)				8.6 (8)	8 (2)				8.6 (3)	18.2 (4)	100 (1)	12.5 (1)		20 (1)					1.2 (1)	40 (4)			
TEM$_{Pen}$/SHV$_{Pen}$/CTX-M/OXA$_{Pen}$/PMQR							4 (1)					9.1 (2)									20 (2)				
TEM$_{Pen}$/SHV$_{Pen}$/CTX-M/OXA$_{Pen}$/OXA$_{Carba}$/PMQR												22.7 (5)													
TEM$_{Pen}$/CTX-M/OXA$_{Pen}$/OXA$_{Carba}$/PMQR												27.3 (6)				50 (1)									
CTX-M/OXA$_{Pen}$/PMQR	8.3 (4)					14 (13)	12 (3)				17.1 (6)					20 (1)					3.5 (3)				
TEM$_{Pen}$/SHV$_{Pen}$/OXA$_{Carba}$/PMQR														37.5 (3)											
CTX-M/PMQR						2.2 (2)		20 (1)			5.7 (2)			12.5 (1)					33.3 (1)						
CTX-M/OXA$_{Pen}$	8.3 (4)					6.5 (6)	8 (2)				2.9 (1)					20 (1)					8.1 (7)				
Other combinations	2.1 (1)	25 (1)				3.2 (3)	16 (4)					22.7 (5)		12.5 (1)				50 (1)	33.3 (1)		20 (2)				
No resistance gene detected	4.2 (2)					14 (13)	12 (3)		50 (2)		8.6 (3)			12.5 (1)	100 (1)						2.3 (2)				
Single resistance gene	20.8 (10)	25 (1)	66.7 (2)			21.5 (20)	28 (7)	80 (4)	25 (1)	100 (1)	22.9 (8)			12.5 (1)		20 (1)				1	23.3 (20)	10 (1)			75 (3)
Total no. of isolates	48	4	3	0	0	93	25	5	4	1	35	22	1	8	1	5	2	0	3	1	86	10	0	0	4

EC, *Escherichia coli*; KP, *Klebsiella pneumoniae*; KO, *Klebsiella oxytoca*; EN, *Enterobacter cloacae*; OS, *Other species*.
[a]TEM$_{Pen}$, TEM Penicillinase, OXA$_{Pen}$, OXA Pencillinase, OXA$_{Carba}$, OXA Carbapenemase, SHV$_{Pen}$, SHV Penicillinase.
[b]Other species include *Enterobacter intermedius* (n = 2). *Enterobacter gergoviae* (n = 1), *Citrobacter freundii* (n = 1), and *Proteus mirabilis* (n = 1), *Providencia stuartii* (n = 1), *Morganella morganii* (n = 1).

Table 5 Distribution of *E. coli* phylogenetic groups among human and animal isolates (n = 267)

Source	No. of isolates	% of phylogenetic groups (no. of isolates)			
		A	B1	B2	D
Human					
Outpatients	48	31.3 (15)	29.2 (14)	25 (12)	14.6 (7)
Inpatients	93	30.1 (28)	26.9 (25)	26.9 (25)	16.1 (15)
Total	141	30.5 (43)	27.7 (39)	26.2 (37)	15.6 (22)
Animal					
Dogs	35	48.6 (17)	22.9 (8)	2.9 (1)	25.7 (9)
Cats	5	60 (3)	20 (1)		20 (1)
Horses	86	40.7 (35)	41.9 (36)	2.3 (2)	14 (12)
Total	126	43.7 (55)	35.7 (45)	2.4 (3)	17.5 (22)
Total no. of isolates	267	36.7 (98)	31.5 (84)	14.9 (40)	16.5 (44)

that the combination of AmpC production and porin loss can result in reduced susceptibility to carbapenems [29]. In contrast to these results, 19 (47.5%) of the 40 examined animal isolates harboured an OXA-48 carbapenemase.

Among 361 isolates resistant to one or more third generation cephalosporins 84% harboured a CTX-M type enzyme. Predominant ESBL genes in human and animal samples were $bla_{CTX-M-15}$ and $bla_{CTX-M-1}$. In the past $bla_{CTX-M-15}$ was merely associated with isolates from humans [30] and CTX-M-1 was the major CTX-M subtype in cattle and pigs in Europe [31]. In addition $bla_{CTX-M-1}$ was also detected in companion animal and poultry isolates [32,33]. In the meantime, attribution of the above-named CTX-M types as specific to either
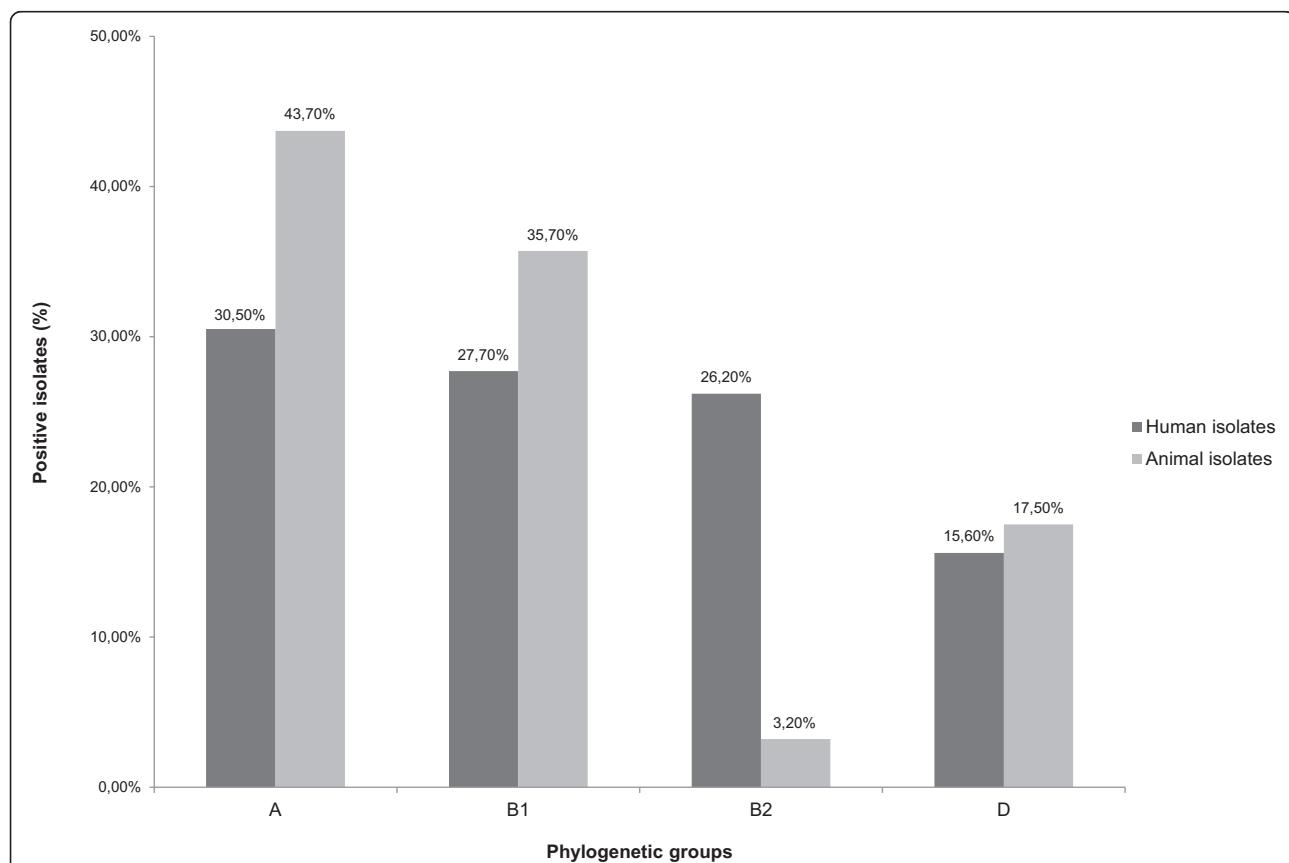

Figure 2 Distribution of *E. coli* phylogenetic groups among human and animal ESBL-producing isolates. A total of 267 isolates from human (n = 141) and animal (n = 126) sources were assigned to *E. coli* phylogenetic groups A, B1, B2 and D.

human or animal isolates is no longer tenable. An increasing number of studies have identified $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$ in both populations [3,34] and our study confirmed this development. Among our isolates $bla_{CTX-M-15}$ was the most prevalent ESBL gene which occurred in approximately 50% of the strains investigated (human: 52.5%, animal: 46.1%). The number for $bla_{CTX-M-1}$ was considerably lesser but this gene was still relatively frequently present (23%, 28.7%). Isolates collected from horses seemed to hold a special position since rates of $bla_{CTX-M-1}$- and $bla_{CTX-M-15}$-positive isolates were almost equal ($bla_{CTX-M-1}$ 37%; $bla_{CTX-M-15}$ 38%). Previous studies have mainly identified CTX-M-1 as the predominant ESBL subtype among horse isolates [35,36], which is also the most common type among livestock. In Germany horses are mostly used as recreational animals, normally situated close to livestock or in livestock-like environments. However, they usually have closer contact to humans and in case of disease medical care for horses is much more intense than in agricultural animal species. This could be a possible explanation for the particularly high detection rates of $bla_{CTX-M-15}$ reported here. Interestingly, $bla_{CTX-M-2}$ was found almost exclusively in equine *E. coli* isolates and was not present among human isolates. A study from Belgium identified $bla_{CTX-M-2}$ as major CTX-M subtype among diseased horses [37]. These animals had received prolonged treatment with several antibiotics and the $bla_{CTX-M-2}$ gene was part of the novel complex class 1 integron ISCR_1. Ferreira et al. could demonstrate the same linkage between $bla_{CTX-M-2}$ and ISCR_1 however, within the chromosome of *E. coli* isolates from healthy broiler chickens in Brazil [38]. Further investigation of the region surrounding the $bla_{CTX-M-2}$ gene may be necessary to identify possible associations with ISCR_1 among the isolates examined in this work. However, the CTX-M-2 subtype is not restricted to animal or even horse isolates. It was previously found in *Salmonella enterica* isolates from the UK and *E. coli* isolates from Brazil originating from clinically ill human patients [39,40].

Ciprofloxacin-resistant animal isolates (55.7%) showed higher prevalence of PMQR-encoding genes compared to human isolates (33.6%). In addition, four *qnr*-variants (*qnrA, qnrB, qnrD* and *qnrS*) were identified in the group of animal isolates, compared to only *qnrB* and *qnrS* among the human isolates. All identified *qnr* variants have already been identified in both groups, however no study is consistent with the results found here [41,42].

Another interesting result is the low prevalence of bla_{SHV-1} among the *K. pneumoniae* isolates (10.3% of the human and in 29.4% of the animal *K. pneumoniae* isolates). Even though it is widely considered a plasmid-encoded enzyme, it has been found at high frequencies in *K. pneumoniae*. Further the bla_{SHV-1} origin is suspected in

the chromosome of *K. pneumoniae* [43]. As some authors indicate high frequencies of up to 90% in *K. pneumoniae* isolates, our data does not seem to fit into the picture [43]. Yet there are other studies showing also low prevalences [44]. Based on these data it could be a local phenomenon.

The β-lactamase genes bla_{SHV-1}, bla_{SHV-5}, bla_{SHV-11} and bla_{TEM-52} were restricted to isolates from inpatients. This fact could be explained by their ancestry, since these genes are frequently found in isolates from hospitals [45].

Combination of various resistance genes was the rule rather than the exception (Table 4). In 70.1% of all isolates two or more genes responsible for β-lactam or quinolone resistance were identified. Combinations of several β-lactamase genes are commonly observed regularly within genomes of Gram-negative bacterial pathogens [46,47] and co-selection of β-lactamase genes and determinants for resistance against other antibiotics e.g. fluoroquinolones has been postulated as a possible mechanism responsible for the widespread distribution of those combined genes [48]. The frequently observed combination of $bla_{CTX-M-15}$ and *aac('6)-Ib-cr* could provide genetic support for this theory, which has been noted before [49,50]. For this study the presence of bla_{OXA-48} in combination with at least two penicillinase genes e.g. TEM, SHV or OXA, a PMQR gene and even sometimes with a CTX-M gene is alarming. Even more worrying is the fact that almost all isolates identified with the OXA-48 carbapenemase originate from companion animals. However, other carbapenemases could be present among the human and animal isolates, which were not tested for. The carbapenem-resistant animal isolates were mainly *K. pneumoniae* and *Enterobacter cloacae* isolates. An unrelated study conducted in our geographic region also detected carbapenem-resistant *Klebsiella* spp. among isolates from dogs [26].

E. coli and *K. pneumoniae* are the main species associated with β-lactamase production among isolates resistant to third generation cephalosporins in Germany and other members of the European Union [51]. Data from this study underlines this predominance of both bacterial species as a carrier of ESBL-encoding resistance genes with 74% *E. coli* and 17.5% *K. pneumoniae* isolates in total. Interestingly, the phylogenetic group B2 was strongly underrepresented among the ESBL-*E. coli* isolates from animals. Compared to the human isolates (26.2%) it was only present in 2.4%. The origins of primary sample material could provide an explanation for this discrepancy. Several studies suggest dominance of certain phylogenetic groups in particular specimen and types of infection [52,53]. Zhang et al. demonstrated clustering of phylogenetic group B2 among urinary *E. coli* isolates from human patients [52]. The fact that 53% of the human

isolates in this study had been recovered from urine while only 19% were from rectal swabs suggests an association between higher numbers of B2-*E. coli* isolates and the urinary tract of humans. The main source for the animal isolates was faeces (51%) from animals suffering from enteritis, diarrhoea and colic. It is not known whether the ESBL isolates detected were indeed causative for the syndromes observed as they could also be commensal strains that were selectively isolated solely on the basis of their resistance phenotype. This could explain the strong presence of phylogenetic group A/B1 and almost complete lack of group B2. Nevertheless, the pathogenic potential of ESBL-encoding isolates remains uncertain and needs further research. Studies performed on *E. coli* harbouring β-lactamases revealed several virulence-associated traits such as toxins, fimbriae, siderophores, polysaccharide coatings and invasins [54,55]. In addition, patients with bacteremic urinary tract infections (UTIs) caused by ESBL-producing *Enterobacteriaceae* also have prolonged hospitalization and higher antibiotic costs [56].

Our study has limitations with regard to the selection of isolates. Human isolates originate from screening of patients upon admission for carrier-status as well as from clinically ill patients, whereas animal isolates only derive from diseased animals. In addition, selection criteria for ESBL- or PMQR-carrying isolates might have introduced bias. A screening breakpoint of >1 mg/L is recommended for cefotaxime in accordance with the guidelines issued by EUCAST [9]. Usage of the higher concentration of 2 mg/L might have led to loss of some ESBL types. PMQR-genes typically induce low-level quinolone-resistance with ciprofloxacin MIC of ≥ 0.25 μg/mL [57]. For this reason some PMQR may not have been detected due to the MIC of 1 μg/mL used.

Notwithstanding, the study gives an overall view on current dissemination of ESBL-encoding *Enterobacteriaceae* isolates within the region investigated. Further studies should be directed to examining specific transmission pathways assessing the exchange between the human and animal populations in owner-pet studies. In order to address this aspect in more detail, simultaneous screening of pets and/or owners positive for ESBL-producing *Enterobacteriaceae* should be carried out in the future. Another important factor for possible transmission or spread is the environment. Multiresistant bacteria are isolated with increasing frequencies from soil or water and wildlife [58]. Some studies implicate ESBL-producing *E. coli* already as a form of environmental pollution [59]. Our findings are supported by other non-regional studies that confirm the data presented [4,60,61]. In line with this work several studies describe presence of ESBL producing *Enterobacteriaceae* among human and animal samples with dominance of the CTX-M subtype [1-3].

Other aspects such as the colonisation and persistence in humans, animals and the environment as well as host species adaption and interspecies transmissibility of the bacteria involved must be taken into account. As plasmids are epidemiologically relevant agents in ESBL transfer, their role and association with sequence types (STs) need to be examined. Transfer of similar plasmids from human to animal bacterial populations and vice versa has been shown in broilers and broiler farmers in the Netherlands [62] and suggest that a similar situation could also exist between pet and owner.

Conclusions

The results from this study substantiate the alarming occurrence and ongoing spread of various ESBL-producing, multiresistant *Enterobacteriaceae* strains in the human and animal populations within a defined geographic region in central Hesse, Germany. Our data confirm that companion animals represent novel reservoirs of carbapenemase-producing strains of *K. pneumoniae* and *Enterobacter cloacae*. The study strongly supports the One Health concept which expands interdisciplinary collaborations and communications in all aspects pertaining to the health care for humans and animals [63].

Ethical approval

The study was approved by the ethics committee of medical faculty of the Justus Liebig University of Giessen (AZ: 95/11).

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