Emergence of OXA-48 carbapenemase-producing Escherichia coli and Klebsiella pneumoniae in dogs

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Objectives: To evaluate the possible occurrence of carbapenemase-producing *Escherichia coli* and *Klebsiella* spp. strains in domestic animals.

Methods: Veterinary clinical *E. coli* (n = 1175) and *Klebsiella* spp. (n = 136) isolates consecutively collected from livestock and companion animals in Germany from June 2012 to October 2012 were screened for their susceptibility to carbapenems using the agar disc diffusion test. Carbapenemase genes were characterized by PCR and sequencing; conjugation assays were performed. Carbapenemase-positive isolates were assigned to phylogenetic lineages by multilocus sequence typing and the clonal relatedness was determined using macrorestriction analysis and subsequent PFGE.

Results: Carbapenem non-susceptible isolates of *Klebsiella pneumoniae* (n=5) and *E. coli* (n=3) were obtained from six dogs hospitalized in a single veterinary clinic in Hessia, Germany, partly at the same time and consecutively over the study period. All isolates harboured carbapenemase gene bla_{OXA-48} located within Tn1999.2 transposons on conjugative ~60 kb plasmids. The *K. pneumoniae* isolates belonged to sequence type ST15, pulsotype 1, and coexpressed CTX-M-15, SHV-28, OXA-1 and TEM-1. Two *E. coli* isolates were assigned to ST1196 and pulsotype 2 and coproduced CMY-2, SHV-12 and TEM-1, while the third *E. coli* isolate was of ST1431 (pulsotype 3), and possessed $bla_{CTX-M-1}$, bla_{OXA-2} and bla_{TEM-1} .

Conclusions: This is the first known report of OXA-48-producing bacteria from companion animals. The clonal nature of the *K. pneumoniae* and two *E. coli* isolates suggests a nosocomial dissemination rather than repeated introduction by individual patients into the clinic.

Keywords: carbapenemase, companion animal, dog, sequence type ST15

Introduction

The most prevalent mechanism of carbapenem resistance among Enterobacteriaceae is the acquisition of carbapenem-hydrolysing enzymes. Commonly recognized carbapenemases are KPC-, NDMand OXA-48-type enzymes, whose encoding genes are located on conjugative plasmids that are easily transferable between different enterobacterial species.¹ Owing to the emergence of OXA-48producing Enterobacteriaceae, first in Turkey and afterwards in several other European and non-European countries in the last five years, these bacteria have received increased attention, in particular in hospital facilities as they are frequently associated with serious therapeutic failures.^{2,3} Multidrug resistance in OXA-48 carbapenemase-producing Enterobacteriaceae often results from the coproduction of various β -lactamases, e.g. extended-spectrum β -lactamases (ESBLs) and plasmid-encoded AmpC β -lactamases, and other resistance determinants.⁴ Recently, OXA-48-type carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* isolates have been isolated in different German hospitals, thus extending the range of countries reporting the emergence of such bacteria.⁵ Reports on carbapenemase-producing bacteria in animals are very rare and so far limited to livestock. Several VIM-1 carbapenemase-producing *E. coli* and *Salmonella* isolates were isolated from pigs in Germany,^{6,7} one *Acinetobacter baumannii* isolate expressing carbapenemase OXA-23 was recently recovered from cattle in France,⁸ and NDM-1-producing *A. baumannii* and

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Acinetobacter Iwoffii isolates were reported from porcine and chicken sources in south China.⁹ Here, we describe the detection and molecular characterization of OXA-48 carbapenemase-producing *K. pneumoniae* and *E. coli* strains obtained from or along with clinical samples from dogs in a veterinary clinic in Germany in 2012.

Materials and methods

Bacterial isolates and antimicrobial susceptibility testing

Between June 2012 and October 2012, specimens (n=3885) from urine (n=382), faeces (n=463), skin and soft tissue (n=563), genital tract (n=534), respiratory tract (n=379), internal organs (n=182) and various other sites (n=1382) were received from veterinary surgeons and clinicians and processed by standard culture methods. All isolated *E. coli* (n=1175)and Klebsiella spp. (n=109 K. pneumoniae; n=27 Klebsiella oxytoca) were screened for susceptibility to carbapenems using imipenem and ertapenem disc diffusion testing as recommended by the CLSI.^{10,11} Phenotypic confirmation of carbapenemase production was performed using the modified Hodge test.¹² MIC data were generated by the VITEK[®]2 system (bioMérieux, Germany; cards AST-N118 and GN-38). Carbapenem-non-susceptible isolates were further tested phenotypically for ESBL production by combination disc tests using ceftazidime and cefotaxime with and without clavulanic acid (Oxoid, Wesel, Germany) according to CLSI guidelines.¹¹ In addition, a cefoxitin disc (30 µq, Oxoid) was used to detect AmpC phenotypes. All isolates classified as intermediate or resistant to cefoxitin according to CLSI criteria $(\leq 17 \text{ mm})$ were suspected to be AmpC producers.

Identification of β -lactamase genes and other resistance genes

Screening for β -lactamase genes bla_{OXA-48} , bla_{VIM} , bla_{NDM-1} , bla_{IMP} and bla_{KPC} , bla_{CTX-M} , bla_{OXA} , bla_{SHV} and bla_{TEM} , bla_{CMY} , bla_{MOX} , bla_{FOX} , bla_{CIT} and bla_{DHA} was carried out using PCR and subsequent sequencing according to previous protocols.^{4,5,13,14} The genetic environment of bla_{OXA-48} was investigated using PCR and sequencing as described previously.¹⁵ The identification of genes *qnr*-like, *qepA*, *oqxA/B* and *aac(6')-Ib* encoding plasmid-mediated quinolone resistance (PMQR) determinants was carried out as described previously.^{14,16}

Conjugation experiments and plasmid characterization

Transferability of β -lactam resistance by conjugation was tested by broth mating assays using a sodium azide-resistant *E. coli* J53 recipient.¹⁷ Plasmids were characterized using S1 nuclease restriction of genomic DNA and subsequent PFGE,¹⁸ as well as PCR-based replicon typing.¹⁹

Molecular typing of bacterial isolates

Bacterial strain typing of *K. pneumoniae* and *E. coli* isolates was performed using XbaI-macrorestriction analysis following a published protocol¹⁴ with interpretation according to the criteria of Tenover *et al.*²⁰ Macrorestriction patterns were digitally compared with a collection of previously typed OXA-48-producing isolates from human patients, using BioNumerics Version 6.6 (Applied Maths, Sint-Martens, Belgium).⁵

Multilocus sequence typing (MLST) of *K. pneumoniae* and *E. coli* isolates was carried out according to published protocols.^{21,22} For gene amplification and sequencing, we used primers specified on the *K. pneumoniae* (www.pasteur.fr/mlst) and *E. coli* MLST web sites (http://mlst.ucc.ie/mlst/ mlst/dbs/Ecoli). DNA sequencing was carried out by an external company (LGC Genomics Berlin, Germany) and sequences were analysed using Ridom Seqsphere (Ridom GmbH, Muenster, Germany).

Results and discussion

During a 5 month period in 2012, we isolated carbapenem nonsusceptible K. pneumoniae subsp. pneumoniae (n=5) and E. coli (n=3) isolates from six doas admitted to a small-animal veterinary clinic in Hessia, Germany (Table 1). The first isolates (K. pneumoniae IHIT 22059 and E. coli IHIT 22060) were recovered from a wound swab of an 11-year-old bulldog with a cardiac pacemaker, exhibiting several comorbidity factors. One day later, bacteriological examination was performed on bronchoalveolar lavage fluid taken from an 8-month-old German shepherd mixed breed dog with eosinophilic pneumonia. Here again, one K. pneumoniae (IHIT 22061) and one E. coli (IHIT 22062) with carbapenem non-susceptibility (Table 2) were isolated. From 21 August to 6 October 2012, another three K. pneumoniae isolates and one E. coli isolate showing carbapenem non-susceptibility were isolated from tracheal washing (IHIT 22063), faeces (IHIT 22064), urine (IHIT 22065) and central venous catheter (IHIT 22066) samples taken from four individual dogs, as shown in Table 1. Besides carbapenem non-susceptible Enterobacteriaceae, other bacterial species, including methicillin-resistant Staphylococcus pseudintermedius (MRSP), A. baumannii, Streptococcus spp., Enterococcus spp. and ESBL-producing E. coli were recovered from the different sample materials. Thus, with the exception of dog 5 suffering from cystitis, where K. pneumoniae was obtained in pure culture, all the other dogs showed mixed bacterial populations in the samples (Table 1).

All eight clinical isolates were positive for bla_{OXA-48}. So far, carbapenemase-producing bacterial isolates have been isolated only occasionally from different livestock animals in Germany,^{6,7} France⁸ and China.⁹ However, there has been no report on carbapenemases in companion animals and, to our knowledge, none about OXA-48-producing bacteria from an animal source at all. Previous studies on human OXA-48-producing isolates indicate that plasmids carrying the *bla*_{OXA-48} gene from different Enterobacteriaceae isolates, clonal groups and geographical regions may share similar characteristics.^{2,4,23} Common features included self-transferability by conjugation, plasmid size (60-70 kb), absence of additional resistance determinants and the failure to determine the replicon type using the PCR-based replicon typing method.¹⁹ These criteria match the results we obtained in the present study in many aspects (Table 2). All *bla*_{OXA-48}-carrying plasmids were transferable by conjugation, had a size of \sim 60 kb and were determined to be of IncL/M-1 replicon type, as previously described.⁴ The detection of Tn1999.2, disrupted by IS1R upstream of bla_{OXA-48} in the animal-derived isolates, hinted towards relevant overlaps with bla_{OXA-48}-carrying plasmids identified in isolates from human patients in Germany and other countries.^{2,5,24} This was further confirmed by the regular detection of the three genes repA, traU and parA in all strains and transconjugants, thus sharing further common features with other sequenced IncL/M plasmids from human sources, such as pHK-NDM and pOXA-48a.^{24,2}

Besides OXA-48, the present isolates coexpressed ESBLs of the CTX-M-15 (*K. pneumoniae*, n=5) or CTX-M-1 type (*E. coli*, n=1), as well as SHV-12 (*E. coli*, n=2) and/or AmpC β -lactamase CMY-2 (*E. coli*, n=2). Furthermore, different PMQR genes (qnrB2, n=2; aac(6')1b-cr, n=8; and oqxA, n=5) and other β -lactamase genes (bla_{OXA-1} , bla_{TEM-1} and bla_{SHV-28}) were identified (Table 2). Transconjugants received from all clinical isolates in this study harboured either the 60 kb bla_{OXA-48} -carrying plasmid alone or in addition to

Dog breed (age at study time)	Bacterial isolate	Isolation date (dd/ mm/yyyy)	Sample material	treatment within 4 weeks prior to bacterial isolation	Other bacteria isolated from the same sample material (unless otherwise stated)	Comorbidity factors/ diagnosis	
Bulldog (11 years)	K. pneumoniae IHIT 22059; E. coli IHIT 22060	09/06/2012	swab (wound tibia)	AMP, AMC	A. baumannii, Streptococcus canis, S. pseudintermedius (MRSP)	chronic otitis, mammary tumour, ventricular tachycardia,	
German shepherd mix (8 months)	K. pneumoniae IHIT 22061; E. coli IHIT 22062	11/06/2012	bronchoalveolar lavage	AMC, ENR	α-haemolytic <i>Streptococcus</i> sp.	eosinophilic pneumonia	
Boxer/Ridgeback (6 years)	K. pneumoniae IHIT 22063	21/08/2012	tracheal washing (after insertion of oesophageal probe)	СТХ	A. baumannii, Enterococcus sp.	macroadenoma pituitary gland	
Yorkshire terrier (3 years)	<i>E. coli</i> IHIT 22064	25/09/2012	faeces (examined to recover E. rhusiopathiae previously isolated from urine)	AMP, AMC, ENR	α-haemolytic Streptococcus sp., Enterococcus sp., Erysipelothrix rhusiopathiae (urine), K. pneumoniae (throat; non-ESBL, non-OXA-48)	portosystemic shunt, cystitis	
Hovawart (2 years)	K. pneumoniae IHIT 22065	25/09/2012	urine	AMP, AMC, ENR	none	portosystemic shunt, cystitis	
Labrador/Retriever (7 years)	K. pneumoniae IHIT 22066	06/10/2012	central venous catheter	AMC, ENR, DOX	A. baumannii, E. coli (ESBL; urine and central venous catheter), Morganella morganii, Citrobacter freundii	suspicion of immune-mediated vasculitis, cystitis, partial splenic torsion	

Table 1. Clinical data from six dogs with confirmed carbapenem non-susceptible Enterobacteriaceae isolates

Dog

no.

1

2

3

4

5

6

AMP, ampicillin; AMC,

		Resistance phenotype	Carbapenem susceptibility							
Date of isolation/ Dog	Bacterial species/isolate no.		mg/L IPM/MEM	mm IPM/MEM/ ETP	Multilocus sequence type (ST)	Pulsotype (XbaI)	β-Lactamases	PMQR genes	Plasmid sizes (kb)	Transferred plasmids, genes (replicon types)
11/06/ 2012 Dog 1	K. pneumoniae IHIT 22059	β-lactams ^a AMK GEN TOB CIP ENR LVX MBX SXT	2/1	22/28/25	ST15	1	OXA-48, CTX-M-15, SHV-28, TEM-1, OXA-1	aac(6′)-Ib-cr, oqxA	50, 60, 75, 100	60 kb, <i>bla</i> _{OXA-48} (IncL/M-1)
	<i>E. coli</i> IHIT 22060	β-lactams ^{a,b} GEN TOB CIP ENR LVX MBX TET CHL SXT	≤1/≤0.25	26/27/22	ST1196	2	OXA-48, CMY-2, TEM-1, SHV-12	aac(6′)-Ib-cr, qnrB2	60, 130, 290	60 kb, <i>bla</i> _{OXA-48} (IncL/M-1)
12/06/ 2012 Dog 2	K. pneumoniae IHIT 22061	β-lactams ^a AMK GEN TOB CIP ENR LVX MBX SXT	2/1	20/28/26	ST15	1	OXA-48, CTX-M-15, SHV-28, TEM-1, OXA-1	aac(6′)-Ib-cr, oqxA	50, 60, 75, 100	60 kb, <i>bla</i> _{OXA-48} (IncL/M-1)
-	E. coli IHIT 22062	β-lactams ^{a,b} AMK GEN TOB CIP ENR LVX MBX TET CHL SXT	≤1/≤0.25	21/26/25	ST1196	2	OXA-48, CMY-2, TEM-1, SHV-12	aac(6')-Ib-cr, qnrB2	60, 130, 290	60 kb, <i>bla</i> _{OXA-48} (IncL/M-1); 130 kb, <i>bla</i> _{TEM-1} (IncFIB); <i>qnrB2</i>
22/08/ 2012 Dog 3	K. pneumoniae IHIT 22063	β-lactams ^a GEN TOB CIP ENR LVX MBX SXT	2/1	24/24/20	ST15	1	OXA-48, CTX-M-15, SHV-28, TEM-1, OXA-1	aac(6′)-Ib-cr, oqxA	50, 60, 75, 100	60 kb, <i>bla</i> _{OXA-48} (IncL/M-1)
25/09/ 2012 Dog 4	<i>E. coli</i> IHIT 22064	β-lactams ^a CIP ENR MBX SXT	<1/≤0.25	26/26/21	ST1431	3	OXA-48, CTX-M-1, TEM-1, OXA-2	aac(6′)-Ib-cr	50, 60, 80, 130	60 kb, <i>bla</i> _{OXA-48} (IncL/M-1); 80 kb, <i>bla</i> _{CTX-M-1} (IncFIB)
25/09/ 2012 Dog 5	K. pneumoniae IHIT 22065	β-lactams ^a GEN TOB CIP ENR LVX MBX TET(I) SXT	8/8	11/14/0	ST15	1	OXA-48, CTX-M-15, SHV-28, TEM-1, OXA-1	aac(6′)-Ib-cr, oqxA	50, 60, 75, 100	60 kb, <i>bla</i> _{OXA-48} (IncL/M-1)
06/10/ 2012 Dog 6	K. pneumoniae IHIT 22066	β-lactams ^a GEN TOB CIP ENR LVX MBX SXT	2/1	25/25/21	ST15	1	OXA-48, CTX-M-15, SHV-28, TEM-1, OXA-1	aac(6′)-Ib-cr, oqxA	50, 60, 75, 100	60 kb, <i>bla</i> _{OXA-48} (IncL/M-1)

Table 2. Phenotypic and genotypic characteristics of OXA-48 carbapenemase-producing Enterobacteriaceae isolates from six dogs

AMK, amikacin; ENR, enrofloxacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; LVX, levofloxacin; MBX, marbofloxacin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; CHL, chloramphenicol; IPM, imipenem; MEM, meropenem; ETP, ertapenem.

^aMIC tests were performed for the following β-lactams: penicillins (ampicillin, piperacillin and ticarcillin); first- to fourth-generation cephalosporins (cefalexin, cefoxitin, cefuroxime, cefoodoxime, cefotaxime, ceftiofur, cefpirome and cefepime); and β-lactamase inhibitors (amoxicillin/clavulanic acid, ampicillin/sulbactam and piperacillin/tazobactam). ^bOnly susceptible to fourth-generation cephalosporins. OXA-48 carbapenemase in Enterobacteriaceae in dogs

a ~130 kb plasmid carrying $bla_{\text{TEM-1}}$ (transconjugant from *E. coli* IHIT 22062 and IHIT 22064). All transconjugants were resistant to ampicillin and ertapenem, but remained susceptible to third-generation cephalosporins (cefotaxime and ceftazidime). The MIC range of the transconjugants was 1–2 mg/L for imipenem and 0.25–1 mg/L for meropenem, respectively. The two transconjugants positive for bla_{OXA-48} and $bla_{\text{TEM-1}}$ were additionally resistant to sulfamethoxazole/trimethoprim and the transconjugant of *E. coli* IHIT 22062 showed increased MIC values for ciprofloxacin (MIC_{CIP}=0.25 mg/L). Subsequently performed PCR revealed the presence of PMQR gene *qnrB2* in this transconjugant.

MLST and macrorestriction analysis showed a clonal spread of K. pneumoniae sequence type ST15 pulsotype 1 with CTX-M-15 and of E. coli ST1196 pulsotype 2 with CMY-2 and SHV-12. For dogs 1 and 2, these clones emerged in combination, whereas dogs 3, 5 and 6 only harboured ST15 K. pneumoniae (Table 2). In contrast to the ST1196 E. coli isolates, the E. coli strain isolated from dog 4 had a different resistance profile, harboured CTX-M-1 instead of CTX-M-15 and did not possess *gnrB2*. Here, we identified a distinct PFGE type (pulsotype 3), and sequence type ST1431, which differed in five out of seven alleles from ST1196. At this time, one can only speculate whether our finding reflects an ongoing *in vivo* transfer of the bla_{OXA-48} -carrying plasmid to other, so far non-affected, bacterial strains and species or the spread of a novel strain that is already bla_{OXA-48} positive. None of the sequence types identified among the canine E. coli strains resembled previously reported OXA-48-producing clones, like ST38²⁴⁻²⁶ and ST131.^{24,27} According to the literature and data available from the E. coli MLST database, only a single ST1196 strain with unknown antimicrobial resistance status has been described so far, obtained from human urine in Korea in 2007. In case of ST1431, two isolates, also lacking any information about their resistance phenotype, were recovered from human patients in Sweden and the UK in 2006 and 2009, respectively (http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli). Notably, we obtained an ST1431 isolate (VB 946937) encoding a CTX-M-1 β-lactamase from a dog with diarrhoea in Germany in 2009 (C. Ewers, S. Guenther, I. Stamm, L.H. Wieler, A. Bethe; unpublished data), suggesting that this ESBL-producing clone might circulate among companion animals.

Concerning *K. pneumoniae*, different clones have been involved in the spread of ESBLs and carbapenemases, such as the epidemic ESBL clone ST15-CTX-M-15,²⁸⁻³² the globally emerging KPC-producing clone ST258^{26,33,34} and OXA-48 carbapenemases-producing clones ST11^{35,36} and ST395.³⁷ Recently, a *K. pneumoniae* ST15 isolate possessing *bla*_{OXA-48} and *bla*_{TEM-1} was identified along with faecal screening of a human patient in Finland, albeit with history of travel to Thailand, suggesting that strains of this sequence type are probably prone to acquire different resistance plasmids.²⁶ The previous detection of *K. pneumoniae* ST15 with CTX-M-15, TEM-1 and OXA-1 in veterinary clinic-acquired urinary tract infections in cats and dogs in France further underlines that this clone might be on its way to extend its global success from the medical to the veterinary area.³⁸

In the USA and Europe, an estimated proportion of 8% of all nosocomial infections in humans are caused by *K. pneumoniae.*²⁹ Mainly due to the spread of Enterobacteriaceae strains that produce ESBLs, carbapenems have become antimicrobial agents of last resort and hence these substances are critical for the prevention and treatment of life-threatening infections.³⁹ In Germany, carbapenems are licensed neither for the treatment of livestock

nor for that of companion animals or pets in general. However, for small-animal populations or for rare infectious diseases. effective antimicrobials may simply not be available due to lack of approval and so there is the opportunity for off-label use under specific circumstances.⁴⁰ This, with respect to animal welfare, exclusively refers to life-threatening diseases and, in the case of bacterial infections, those resistant to treatment with cephalosporins and other antimicrobial substances. However, off-label usage of carbapenems is not common practice at the veterinary clinic where we obtained the OXA-48 producers, evidenced by the history of antimicrobial treatment of the dogs, which is documented in Table 1. Although we cannot rule out that carbapenems had been administered to the dogs once in their lifetime, it seems more likely that they had undergone nosocomial colonization rather than experienced in vivo selection of OXA-48-producers driven by antibiotic treatment. This is further supported by the fact that all dogs except for dog 2, which was, however, previously housed together with dog 1, were kept in the intensive care unit for at least 24 h during their stay in the veterinary clinic, hinting towards a common environmental source. Efforts are currently under way to determine whether the maintenance of multidrug-resistant enterobacterial isolates might be owing to an undetected hygiene problem or putative human carriers (veterinary personnel or surgeons). So far, OXA-48-producing isolates have mainly appeared as colonizers rather than as primary or sole aetiological agents, almost always correlated with comorbidities in the patients.

In conclusion, we report the emergence and clonal spread of *K. pneumoniae* and *E. coli* producing carbapenemase OXA-48 in dogs. Since resistance to carbapenems is not commonly tested for in veterinary clinical isolates, non-susceptible isolates are probably underreported. Due to the increasingly frequent finding of carbapenemase-producing Enterobacteriaceae in humans, and their still sporadic occurrence in animals, a transfer from humans to animals appears highly probable. The present findings add to a growing number of reports on multidrug-resistant strains from human and animal sources—a concerning development that needs constant attention and further investigations.

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Transparency declarations

None to declare.

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