J Antimicrob Chemother 2012; **67**: 1368–1374
doi:10.1093/jac/dks049 Advance Access publication 1 March 2012

Journal of Antimicrobial Chemotherapy

Occurrence and characteristics of extended-spectrum-β-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses

C. M. Dierikx[1]*, E. van Duijkeren[2,3], A. H. W. Schoormans[2], A. van Essen-Zandbergen[1], K. Veldman[1], A. Kant[1], X. W. Huijsdens[3], K. van der Zwaluw[3], J. A. Wagenaar[1,2] and D. J. Mevius[1,2]

[1]Central Veterinary Institute (CVI) of Wageningen UR, Department of Bacteriology and TSEs, PO Box 65, 8200 AB Lelystad, The Netherlands; [2]Utrecht University, Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, PO Box 80165, 3508 TD Utrecht, The Netherlands; [3]National Institute for Public Health and the Environment (RIVM), Centre for Infectious Disease Control (CIb), PO Box 1, 3720 BA Bilthoven, The Netherlands

*Corresponding author. Tel: +31-320-238327; Fax: +31-320238153; E-mail: cindy.dierikx@wur.nl

Received 7 September 2011; returned 7 November 2011; revised 12 January 2012; accepted 31 January 2012

Objectives: To investigate the occurrence and characteristics of extended-spectrum β-lactamase (ESBL)- and AmpC-producing Enterobacteriaceae isolates in clinical samples of companion animals and horses and compare the results with ESBL/AmpC-producing isolates described in humans.

Methods: Between October 2007 and August 2009, 2700 Enterobacteriaceae derived from clinical infections in companion animals and horses were collected. Isolates displaying inhibition zones of ≤25 mm for ceftiofur and/or cefquinome by disc diffusion were included. ESBL/AmpC production was confirmed by combination disc tests. The presence of resistance genes was identified by microarray, PCR and sequencing, *Escherichia coli* genotypes by multilocus sequence typing and antimicrobial susceptibility by broth microdilution.

Results: Sixty-five isolates from dogs ($n=38$), cats ($n=14$), horses ($n=12$) and a turtle were included. Six Enterobacteriaceae species were observed, mostly derived from urinary tract infections ($n=32$). All except 10 isolates tested resistant to cefotaxime and ceftazidime by broth microdilution using clinical breakpoints. ESBL/AmpC genes observed were $bla_{\text{CTX-M-1, -2, -9, -14, -15}}$, $bla_{\text{TEM-52}}$, $bla_{\text{CMY-2}}$ and $bla_{\text{CMY-39}}$. $bla_{\text{CTX-M-1}}$ was predominant ($n=17$). $bla_{\text{CTX-M-9}}$ occurred in combination with *qnrA1* in 3 of the 11 *Enterobacter cloacae* isolates. Twenty-eight different *E. coli* sequence types (STs) were found. *E. coli* carrying $bla_{\text{CTX-M-1}}$ belonged to 13 STs of which 3 were previously described in Dutch poultry and patients.

Conclusions: This is the first study among a large collection of Dutch companion animals and horses characterizing ESBL/AmpC-producing isolates. A similarity in resistance genes and *E. coli* STs among these isolates and isolates from Dutch poultry and humans may suggest exchange of resistance between different reservoirs.

Keywords: AmpC β-lactamases, dogs, cats, horses, ESBLs

Introduction

Members of the family Enterobacteriaceae commonly express plasmid-encoded broad-spectrum β-lactamases (TEM-1, TEM-2 and SHV-1) that confer resistance to amino-penicillins and first-generation cephalosporins, but not to third- and fourth-generation cephalosporins. The introduction of third-generation cephalosporins in the 1980s was a milestone in antimicrobial chemotherapy and improved the treatment options in human and veterinary medicine.[1] Unfortunately, resistance to extended-spectrum cephalosporins (ESCs) emerged a few years later. Resistance to ESCs in Enterobacteriaceae is most often related to the production of extended-spectrum β-lactamases (ESBLs) or AmpC β-lactamases. ESBLs confer resistance to amino-penicillins, cephalosporins and monobactams and are inhibited by clavulanic acid. AmpC β-lactamases have a broader spectrum of resistance, including the cephamycins, and are not inhibited by β-lactamase inhibitors. The production of ESBLs and AmpC β-lactamases is often plasmid mediated. Moreover, these plasmids frequently carry genes encoding resistance to other drug classes, such as fluoroquinolones, aminoglycosides, sulfa-derivatives and trimethoprim.[2,3] Therefore treatment options for infections caused by ESBL- and/or AmpC-producing organisms are limited. Initially these organisms were associated with

hospitals and institutional care in humans, but they are now increasingly found in the community and in food-producing animals, particularly poultry, suggesting an exchange of organisms or genes between the different reservoirs, or a different antibiotic use behaviour.[4] In the Netherlands, ESCs are authorized for use in food-producing animals (ceftiofur, cefquinome) and companion animals (cefovecin). Resistance to ESCs has been studied in detail in Gram-negative bacteria isolated from humans and food-producing animals.[5,6] However, data on ESBL- and AmpC β-lactamase-producing Enterobacteriaceae in companion animals and horses are limited.[7] The objective of this study was to investigate the occurrence of ESBL/AmpC-producing organisms in clinical samples, to further characterize these isolates and to compare the results with ESBL- and AmpC-producing isolates described in humans.

Materials and methods

Bacterial isolates

The Veterinary Microbiological Diagnostic Center (VMDC) of Utrecht University investigates samples from all over the Netherlands. Per year approximately 10000 samples are submitted for bacteriological analysis and about 48% of these samples originate from dogs, 18% from cats, 12% from horses, 10% from food-producing animals and 12% from other animals including reptiles, birds and mammals other than those mentioned. From October 2007 to August 2009, 10755 isolates, derived from approximately 18000 samples, of which 2700 were Enterobacteriaceae, were tested for susceptibility to a wide range of antimicrobial agents by disc diffusion using a semi-confluent inoculum of the bacteria on Iso-Sensitest agar (bioTRADING, Mijdrecht, The Netherlands) with Neo-Sensitabs (Rosco Diagnostica, Taastrup, Denmark). Species were identified by conventional biochemical methods. *Salmonella* serovar identification was performed using microtitre and slide agglutination methods (O- and H-group antigens) according to the latest version of the Kauffman–White scheme. All Enterobacteriaceae isolates that displayed an inhibition zone ≤25 mm for ceftiofur (dog/cat/turtle isolates) and/or cefquinome (horse isolates) were included in the study and stored at −80°C until further analysis.

Phenotypic ESBL/AmpC testing

The isolates were tested phenotypically for ESBL production by combination disc tests using cefotaxime and ceftazidime with and without clavulanic acid (Becton Dickinson) according to CLSI guidelines.[8] In addition, a cefoxitin disc (30 μg, Becton Dickinson) was added to this test, to detect AmpC phenotypes. All isolates classified as intermediate or resistant using CLSI criteria (≤17 mm) to cefoxitin were suspected to be AmpC producers.

Escherichia coli genotyping

In order to compare the genotypes of the *E. coli* isolates found in this study with ESBL-producing *E. coli* in former human studies, all *E. coli* strains were genotyped by multilocus sequence typing (MLST) as described previously.[9] Sequences were uploaded on the MLST web site (http://mlst.ucc.ie/).

Susceptibility testing

All isolates were tested for antimicrobial susceptibility by determining MICs using broth microdilution, according to the international standard ISO 20776-1:2006. MICs were determined for ampicillin (concentration range 0.5–32 mg/L), cefotaxime (0.06–4 mg/L), ceftazidime (0.25–16 mg/L), ciprofloxacin (0.008–4 mg/L), nalidixic acid (4–64 mg/L), colistin (2–4 mg/L), gentamicin (0.25–32 mg/L), kanamycin (4–128 mg/L), sulfamethoxazole (8–1024 mg/L), trimethoprim (0.5–32 mg/L), streptomycin (2–128 mg/L), tetracycline (1–64 mg/L), chloramphenicol (2–64 mg/L) and florfenicol (2–64 mg/L) using *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 as control isolates. Multidrug resistance was defined as resistance to three or more antimicrobial agents included in the following list of eight antibiotics: cefotaxime (R >2 mg/L), ciprofloxacin (R >1 mg/L), colistin (R >2 mg/L), gentamicin (R >4 mg/L), sulfamethoxazole (R >256 mg/L), trimethoprim (R >4 mg/L), tetracycline (R >8 mg/L) and chloramphenicol (R >8 mg/L). Isolates were classified as resistant based on EUCAST clinical breakpoints (www.eucast.org) and, if not available (for sulfamethoxazole and tetracycline), CLSI clinical breakpoints.[8] *Serratia marcescens* is intrinsically resistant to colistin and *Proteus mirabilis* to both colistin and tetracycline.[10] These bacterial species were not included to calculate multiresistance.

β-Lactamase identification

All isolates were screened for bla_{SHV}, bla_{LEN}, bla_{TEM}, bla_{OXA}, bla_{CTX-M}, bla_{DHA}, bla_{ACC}, bla_{MOX}, bla_{FOX} or bla_{CMY} gene families by miniaturized microarray (Identibac, AMR-ve 05 genotyping, Alere International, Tilburg, The Netherlands).[11] The gene families that responded positively in the array were further typed by PCR and sequencing using primers displayed in Table 1. PCR consisted of 30 cycles (30 s of denaturation at 94°C, 30 s of annealing at temperatures mentioned in Table 1 and 60 s of extension at 72°C) after one step of 5 min at 94°C. Amplicons were purified and sequenced as described previously.[5] All *E. coli* isolates with AmpC phenotypes that could not be attributed to the presence of a plasmid-mediated AmpC gene were tested for mutations in the *ampC* promoter/attenuator region as described previously.[12] PCRs to detect bla_{SHV}, bla_{CTX-M} or bla_{TEM} genes were performed in isolates with an ESBL phenotype in which the array was negative for probes encoding ESBL gene families (Table 1).

Additional resistance gene identification

Antibiotic resistance genes were detected using the same microarray that was used for the screening of β-lactamase genes. This array included probes for 40 non-β-lactam resistance gene families known to occur in Gram-negative bacteria.[11] Isolates with positive signals for *qnr* genes, which encode for plasmid-mediated quinolone resistance genes, were further analysed by PCR and sequencing using primers for *qnrA*, *qnrB* and *qnrS* as displayed in Table 1 and as described previously.[13]

Results

During the study period (October 2007–August 2009), 2% of all Enterobacteriaceae ($n=65$) displayed inhibition zones ≤25 mm for ceftiofur or cefquinome. Between January 2008 and August 2009, this was found for 3% of the Enterobacteriaceae derived from dogs, 4% from cats and 8% from horses. For 2007 these data were not available. In 2007 a total of eight isolates matched the inclusion criteria. In 2008, 28 isolates were included, and in 2009, 29 isolates. All 65 isolates originated from clinical infections from dogs ($n=38$), cats ($n=14$), horses ($n=12$) and a turtle. Except for two horses, all animals belonged to different owners and were submitted by 37 veterinary clinics located in 11 provinces in the Netherlands. One isolate was cultured from a dog that lived in an animal shelter in Germany. Information about antibiotic treatment was not available for all animals, but given the fact that most veterinary practices send

Table 1. Primers and PCR conditions used in this study to detect ESBL, plasmid-mediated AmpC, chromosomal *ampC* and *qnr* genes

Target	Primer	Annealing temperature (°C)	Sequence (5′-3′)	Product size (bp)	Reference
CTX-M families	CTX-M-F CTX-M-R	55	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYS AGC GG	592	5
CTX-M-1	CTX-1-SEQ-F CTX-1-SEQ-R	60	CCC ATG GTT AAA AAA TCA CTG C CAG CGC TTT TGC CGT CTA AG	>1000	28
CTX-M-2	CTX-M-2F CTX-M-2R	55	ATG ATG ACT CAG AGC ATT CG TTA TTG CAT CAG AAA CCG TG	886	5
CTX-M-9	CTX-M-9-1F CTX-M-4R	55	TGG TGA CAA AGA GAG TGC AAC G TCA CAG GCC TTC GGC GAT	875	20
CTX-M-14/17	CTX-M-9_{792}F CTX-M-9_{1029}R	55	CTA TTT TAC CCA GCC GCA AC GTT ATG GAG CCA CGG TTG AT	238	this study
SHV	SHV-F SHV-R	55	TTA TCT CCC TGT TAG CCA CC GAT TTG CTG ATT TCG CTC GG	796	5
TEM	TEM-F TEM-R	55	GCG GAA CCC CTA TTT G ACC ATT GCT TAA TCA GTG AG	964	5
CMY	CMY-F CMY-R	58	ATG ATG AAA AAA TCG TTA TGC TGC GCT TTT CAA GAA TGC GCC AGG	1138	5
Chromosomal *ampC*	AmpC1_{-71} AmpC2_{120}	55	AAT GGG TTT TCT ACG GTC TG GGG CAG CAA ATG TGG AGC AA	191	12
OXA-1	OXA-1-F OXA-1-R	55	ATG AAA AAC ACA ATA CAT ATC AAC TTC GC GTG TGT TTA GAA TGG TGA TCG CAT T	820	29
OXA-2	OXA-2-F OXA-2-R	55	ACG ATA GTT GTG GCA GAC GAA C ATY CTG TTT GGC GTA TCR ATA TTC	601	29
qnrA	*qnrA*-F *qnrA*-F	53	ATT TCT CAC GCC AGG ATT TG GAT CGG CAA AGG TTA GGT CA	516	13
qnrB	*qnrB*-F *qnrB*-R	53	GAT CGT GAA AGC CAG AAA GG ACG ATG CCT GGT AGT TGT CC	469	13
qnrS	*qnrS*-F *qnrS*-R	53	ACG ACA TTC GTC AAC TGC AA TAA ATT GGC ACC CTG TAG GC	417	13

in their samples only when initial antibiotic treatment fails, the isolates obtained are considered to be derived from animals that have received one or more antibiotic treatments. The isolates were cultured from urine ($n=32$), wound ($n=15$), peritoneal fluid ($n=5$), uterus ($n=5$), trachea ($n=3$), blood ($n=2$), faeces ($n=1$), bile ($n=1$) and a throat sample ($n=1$). Most isolates were *E. coli* ($n=48$). Other isolates were identified as *Enterobacter cloacae* ($n=11$), *P. mirabilis* ($n=3$), *Salmonella enterica* subspecies *enterica* 4,[5],12:b:− ($n=1$), *S. marcescens* ($n=1$) and *Citrobacter freundii* ($n=1$).

Combination disc tests resulted in 29 isolates displaying an ESBL phenotype, 22 an AmpC phenotype, 6 a mixed ESBL/AmpC phenotype (displaying synergy with clavulanic acid and resistance to cefoxitin) and 2 displayed inconclusive results in the confirmation test (resistant to cefotaxime and/or ceftazidime, but no synergy with clavulanic acid and susceptible to cefoxitin). This was designated to be an inhibitor-resistant ESBL (IRE) type. In the remaining six isolates (four from dogs and two from cats), although with inhibition zones of ≤25 mm for ceftiofur, the ESBL phenotypic test was negative. These six isolates had MICs between ≤0.06 and 0.5 mg/L for cefotaxime and between ≤0.25 and 0.5 mg/L for ceftazidime, and molecular analysis of these isolates did not result in the detection of an ESBL and/or AmpC gene. The isolates that showed either an ESBL, ESBL/AmpC or IRE type were all resistant to cefotaxime (MIC >2 mg/L) and/or ceftazidime (MIC >4 mg/L). Four isolates displaying an AmpC phenotype had cefotaxime and ceftazidime MIC values below the clinical breakpoints. In sum, of 2700 isolates screened, 55 isolates (2.0%) were resistant to either cefotaxime or ceftazidime.

In the 29 isolates with an ESBL phenotype the following ESBL genes were found: $bla_{\text{CTX-M-1}}$ ($n=17$), $bla_{\text{CTX-M-15}}$ ($n=4$), $bla_{\text{CTX-M-14}}$ ($n=1$), $bla_{\text{CTX-M-2}}$ ($n=2$), $bla_{\text{TEM-52}}$ ($n=3$) and a combination of $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-14}}$ ($n=1$). In one isolate no ESBL gene could be detected (Table 2). No bla_{SHV} or bla_{OXA} genes encoding for ESBL production were detected.

Table 2. Characteristics of Enterobacteriaceae isolates displaying inhibition zones of ≤25 mm for ceftiofur or cefquinome from clinical infections in companion animals and horses

Phenotype	*bla* and *qnr* genes	Number of isolates	Species	*E. coli* MLST	Year of isolation	Source	Material
ESBL (*n*=29)	CTX-M-1	7	*E. coli*	117, 162, 141, 770, 2030, 2226, 461	2008 (*n*=5), 2009 (*n*=2)	dog (*n*=2), cat (*n*=2), horse (*n*=3)	peritoneal fluid (*n*=2), urine (*n*=2), wound (*n*=1), uterus (*n*=2)
	CTX-M-1, TEM-1	9	*E. coli*	88, 162 (*n*=2), 457, 362, 34 (*n*=3), 58	2008 (*n*=3), 2009 (*n*=6)	dog (*n*=4), horse (*n*=5)	urine (*n*=4), blood (*n*=1), uterus (*n*=3), trachea (*n*=1)
	CTX-M-1, CTX-M-14, TEM-1	1	*E. coli*	1287	2008	horse	wound
	CTX-M-1, TEM-80	1	*E. coli*	461	2009	dog	wound
	CTX-M-14	1	*E. coli*	1287	2008	horse	wound
	CTX-M-15	1	*E. coli*	648	2008	dog	throat
	CTX-M-15, TEM-1	2	*E. coli*	88, 156	2008 (*n*=2)	dog (*n*=2)	urine (*n*=2)
	CTX-M-15, TEM-1, OXA-1	1	*E. coli*	131	2008	dog	urine
	CTX-M-2, TEM-1	2	*E. coli*	156 (*n*=2)	2007, 2008	cat, horse	urine, wound
	TEM-52	3	*E. coli* (*n*=2), *S. enterica* (*n*=1)	58, 93	2007 (*n*=1), 2009 (*n*=2)	dog (*n*=2), cat (*n*=1)	urine (*n*=2), faeces (*n*=1)
	TEM-1, OXA-1	1	*E. coli*	117	2007	dog	peritoneal fluid
AmpC (*n*=22)	CMY-2	6	*E. coli* (*n*=3), *P. mirabilis* (*n*=3)	297, 372, 2227	2008 (*n*=3), 2009 (*n*=3)	dog (*n*=5), cat (*n*=1)	urine (*n*=6)
	CMY-2, OXA-1	1	*E. coli*	88	2007	dog	urine
	CMY-2, SHV-1	1	*E. coli*	12	2008	cat	wound
	CMY-2, TEM-1	2	*E. coli*	68 (*n*=2)	2008, 2009	dog (*n*=2)	urine (*n*=1), wound (*n*=1)
	CMY-39, *qnrB17*var[a]	1	*C. freundii*	NA	2007	turtle	trachea
	chromosomal *ampC* mutations[b]	3	*E. coli*	372, 58, 88	2008 (*n*=1), 2009 (*n*=2)	dog (*n*=2), cat (*n*=1)	peritoneal fluid (*n*=1), bile (*n*=1), urine (*n*=1)
	chromosomal *ampC* type 3[c], TEM-1	1	*E. coli*	539	2009	dog	urine
	TEM-1	1	*E. cloacae*	NA	2009	horse	wound
	TEM-1, *qnrS1/S3*	2	*E. cloacae*	NA	2008	cat (*n*=2)	wound (*n*=2)
	none	4	*E. cloacae*	NA	2008 (*n*=1), 2009 (*n*=3)	dog (*n*=2), cat (*n*=2)	urine (*n*=4)
ESBL/AmpC (*n*=6)	CTX-M-9	1	*E. cloacae*	NA	2008	cat	wound
	CTX-M-9, *qnrA1*	3	*E. cloacae*	NA	2008 (*n*=1), 2009 (*n*=2)	dog (*n*=3)	blood, trachea, urine

Continued

Table 2. *Continued*

Phenotype	*bla* and *qnr* genes	Number of isolates	Species	*E. coli* MLST	Year of isolation	Source	Material
	CTX-M-15, CMY-2, TEM-1	1	*E. coli*	648	2009	dog	wound
	CTX-M-14, TEM-1, chromosomal *ampC* type 4[c]	1	*E. coli*	405	2007	dog	peritoneal fluid
IRE ($n=2$)	TEM-30	1	*E. coli*	88	2007	dog	wound
	none	1	*S. marcescens*	NA	2007	dog	wound
Susceptible ($n=6$)	OXA-1	1	*E. coli*	88	2009	dog	urine
	TEM-1	3	*E. coli*	448, 950, 1642	2008 ($n=1$), 2009 ($n=2$)	dog ($n=3$)	urine ($n=3$)
	TEM-1, OXA-1	1	*E. coli*	1642	2009	cat	wound
	none	1	*E. coli*	2225	2008	cat	urine

IRE, inhibitor-resistant ESBL; *E. coli*, *Escherichia coli*; *S. enteric*, *Salmonella enterica* subspecies *enterica* 4,[5],12:b:−; *P. mirabilis*, *Proteus mirabilis*; *E. cloacae*, *Enterobacter cloacae*; *C. freundii*, *Citrobacter freundii*; *S. marcescens*, *Serratia marcescens*; NA, not applicable.

[a]Sequence is similar to *qnrB17*, but has one silent mutation at position 169 GC**G**→GC**A**.

[b]Chromosomal *ampC* mutations type 2, 3 and 31 according to Mulvey *et al*.[14]

[c]Chromosomal *ampC* types refer to mutations in the chromosomal *ampC* gene as described by Mulvey *et al*.[14]

Among the 22 isolates with an AmpC phenotype, 11 carried an ESBL or AmpC gene [bla_{CMY-2} ($n=10$) and bla_{CMY-39} ($n=1$)] and one of these isolates (a *P. mirabilis* with bla_{CMY-2}) was clinically susceptible to cefotaxime and ceftazidime (Table S1, available as Supplementary data at *JAC* Online). Four isolates with an AmpC phenotype had mutations in the promoter region of the chromosomal *ampC* gene. These four isolates had mutations in the *ampC* gene promoter belonging to sequence types (STs) 2, 3 ($n=2$) and 31 as described by Mulvey *et al*.[14] Two of those isolates (one with type 2 and one with type 3) were clinically susceptible to cefotaxime and ceftazidime (Table S1). The remaining seven isolates displaying an AmpC phenotype all belonged to *E. cloacae*. In these isolates no plasmid-mediated AmpC gene was detected with the methods used (Tables 2 and S1). One isolate of this group had MICs of 1 mg/L and 0.5 mg/L for cefotaxime and ceftazidime, respectively.

Among the isolates showing a combined ESBL/AmpC phenotype, $bla_{CTX-M-9}$ ($n=4$, all *E. cloacae*), $bla_{CTX-M-14}$ in combination with type 4 mutations in the *ampC* attenuator region[14] ($n=1$) and a combination of $bla_{CTX-M-15}$ and bla_{CMY-2} were detected ($n=1$) (Tables 2 and S1).

Among the two isolates with inconclusive results in the combination disc test (IRE), one carried bla_{TEM-30} and in the other isolate (*S. marcescens*) no ESBL or AmpC genes were observed (Table 2).

Most isolates were multidrug resistant: 74% of all 61 isolates included in the calculation (all isolates, except *P. mirabilis* and *S. marcescens*) were resistant to three or more antimicrobial classes, and 32% were resistant to more than five classes of antibiotics. Among the isolates included in this calculation, nine were clinically susceptible to cefotaxime and/or ceftazidime, six of these were resistant to three or more antibiotics and one of them was resistant to more than five antibiotics. Besides β-lactamase genes, the following gene families were observed, encoding resistance to trimethoprim [*dfrA1* (23%), *dfrA12* (5%), *dfrA14* (3%), *dfrA17* (25%), *dfrA19* (2%), *dfrV* (6%)], sulphonamides [*sul1*(42%), *sul2* (12%), *sul3* (2%)], fluoroquinolones [*qnr* (detects *qnrA* genes, 6%), *qnrB* (2%), *qnrS* (3%)], tetracyclines [*tetA* (6%), *tetB* (25%), *tetC* (2%)], aminoglycosides [*aadA1* (29%), *aadA2* (17%), *aadA4* (14%), *aac(6′)-Ib* (8%), *strA* (3%), *strB* (25%), *ant2a* (8%)], chloramphenicol [*cmlA1* (3%), *catA1* (25%), *catB3* (2%)], florfenicol [*floR* (5%)] and macrolides [*ermB* (3%), *ereA* (3%), *ereB* (20%)]. In addition, integrase genes *intI1* (29%) and *intI2* (3%) were found (Table S1). PCR and sequencing of the seven isolates positive for a *qnr* gene in the array demonstrated the presence of three *qnrA1* genes; all three were detected in *E. cloacae* isolates from dogs also carrying $bla_{CTX-M-9}$. Two *qnrS1/S3* genes (no distinction could be made between *qnrS1* and *qnrS3* with the primers used) were observed in *E. cloacae* carrying bla_{TEM-1} isolated from cats and one *qnrB17* variant (*qnrB17* with one silent mutation at position 169 GC**G**→GC**A**) was found in *C. freundii* carrying bla_{CMY-39} isolated from a turtle. In one isolate (ID 32, Table S1), although positive for the *qnr* probe in the array, no *qnr* PCR product could be amplified with the primers used in this study (Table 1). This strain was susceptible to ciprofloxacin (MIC 0.015 mg/L) as well as to nalidixic acid (MIC ≤4 mg/L) (Table S1), confirming the absence of a *qnr* gene.

MLST of the *E. coli* isolates resulted in 28 different STs (Table 2). The predominant STs were ST88 ($n=6$), ST162 ($n=3$),

ST58 ($n=3$), ST34 ($n=3$) and ST156 ($n=3$). Three new STs were identified: ST2225, ST2226 and ST2227. Except for the two isolates from the horses owned by the same person, which both belonged to ST34, no relation was found between STs and the area where the animals lived, or where the isolates were collected (data not shown).

Discussion

A prevalence of 2% ESC resistance in clinical isolates derived from companion animals is comparable to what is found in other countries.[15,16] In this study we used ceftiofur or cefquinome to detect resistance to ESCs. Veterinary diagnostic laboratories often use cephalosporins that are commonly used in veterinary practice to perform susceptibility tests. However, these antibiotics have been shown to be less suitable as indicator cephalosporins for isolates with ESBL or plasmid-mediated AmpC β-lactamases.[17] In our study, screening with these cephalosporins using non-standardized interpretive criteria resulted in 19 (29%) apparently false-positive isolates, in which no ESBL and/or plasmid-mediated AmpC genes could be detected by array or PCR and sequencing. Six of these (all *E. coli*) were negative in the phenotypic confirmation test and had MICs below clinical breakpoints for cefotaxime and ceftazidime. Four other isolates (two *E. coli*, one *P. mirabilis* and one *E. cloacae*), although displaying an AmpC phenotype, also had MICs below the clinical breakpoints for both ESCs. This resulted in a misclassification of 15% (10/65) of the isolates being resistant to ESCs using $\leq$25 mm as cut-off value for ceftiofur or cefquinome in the disc diffusion test with Neo-Sensitabs (Rosco). To have fewer false-positive results in the future both the method and the interpretative criteria should be critically analysed and possibly reconsidered.

Overall a variety of ESBL genes was found within six bacterial species isolated from four animal species. $bla_{\text{CTX-M-1}}$ was the predominant ESC resistance gene found (26%). This gene was also the predominant ESBL gene among Enterobacteriaceae studied in Dutch horses in 2003–05.[18] At that time, only $bla_{\text{CTX-M-1}}$ was isolated. Our study shows that in later years $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-14}}$ also occurred in Enterobacteriaceae from Dutch horses.

$bla_{\text{CTX-M-1}}$ was recently described as the predominant ESBL gene in cefotaxime-resistant *E. coli* and *S. enterica* isolates (49%) of Dutch poultry[5] and in poultry meat isolates (49%) derived from Dutch supermarkets.[6] In Dutch patients, $bla_{\text{CTX-M-1}}$ was not predominant in clinical infections (21%–24%),[6,19] but it was the predominant gene (46%) found in ESC-resistant *E. coli* derived from rectal swabs of human patients in Dutch hospitals.[19] Our results show that $bla_{\text{CTX-M-1}}$ was present among others in *E. coli* ST117 ($n=2$), ST58 ($n=3$) and ST162 ($n=3$). These *E. coli* genotypes are described in Dutch poultry (ST117 and ST58) and patients (ST117, ST58 and ST162) carrying $bla_{\text{CTX-M-1}}$.[6] This suggests a clonal spread of these *E. coli* genotypes carrying $bla_{\text{CTX-M-1}}$ between different hosts.

A remarkable finding in our study was that all $bla_{\text{CTX-M-9}}$ genes ($n=4$) occurred in *E. cloacae* isolates, three derived from dogs and one from a cat. The canine isolates also harboured the plasmid-mediated quinolone resistance gene *qnrA1*. The combined presence of $bla_{\text{CTX-M-9}}$ and *qnrA1* has been described in outbreak strains of *Enterobacter hormaechei* (initially also classified as *E. cloacae*) in a Dutch hospital at Utrecht University Medical Centre carrying a conjugative plasmid pQC of the IncHI2 family with several complex integrons containing *aadB*, $bla_{\text{CTX-M-9}}$ and *qnrA1*.[20,21] The three canine isolates were not screened for the *aadB* gene, but had non-wild-type MICs of gentamicin, and two isolates contained a class 1 integron. To our knowledge this has not been reported previously in clinical isolates from dogs. The dogs and their owners originated from three different areas in the Netherlands and none of them originated from the area around Utrecht, which confirms the observed nationwide outbreak of *qnrA1*-positive multidrug-resistant *E. cloacae* in human patients.[22] Moreover, in 2008 an incHI2 plasmid containing *qnrA1* and $bla_{\text{CTX-M-9}}$ was found in *Salmonella* Paratyphi B var. Java isolated from broilers.[23,24] Together with our data this suggests that a plasmid with this combination of resistance genes is not only present in different areas within the Netherlands, but has also disseminated in different hosts, including humans, food-producing animals and companion animals.

Another interesting finding in our study was the presence of $bla_{\text{CTX-M-15}}$ in different *E. coli* genotypes. Only one isolate derived from a dog carrying $bla_{\text{CTX-M-15}}$ belonged to uropathogenic *E. coli* ST131. This ST has emerged globally in hospital and community settings and has been described frequently in humans[25] and incidentally in isolates derived from dogs and horses in several European countries,[25–27] emphasizing the intraspecies and pan-European spread of this resistant clone. Our data, however, confirm that $bla_{\text{CTX-M-15}}$ in *E. coli* of dogs is not limited to *E. coli* ST131, and that other clones like ST88, ST156 and ST648 play a role in the dissemination of this resistance gene as well.

This is the first study providing information about the genes and genotypes involved in ESBL- or AmpC-producing isolates from Dutch companion animals and horses in a large group of isolates. The most prevalent gene found, $bla_{\text{CTX-M-1}}$, was previously found in Dutch human and poultry isolates. This, together with the presence of the combination of $bla_{\text{CTX-M-9}}$ and *qnrA1* in *E. cloacae* isolates, which was previously described in human isolates, suggests transmission between the different reservoirs or the existence of a common source. The fact that companion animals often live in close contact with their owners makes the occurrence of transmission between them even more likely. This study shows that prudent usage of antibiotics in companion animals and horses should be emphasized and continued susceptibility surveillance to ESCs is recommended.

Acknowledgements

This work was partly presented in an oral presentation at the Scientific Spring Meeting of the Dutch Society for Medical Microbiology and the Dutch Society for Microbiology in Arnhem, The Netherlands, 2011 (abstract number 0194) and in a poster at the Fourth Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE), Tours, France, 2011 (abstract number P55).

We would like to thank the EU Reference Laboratory for Antimicrobial Resistance at the Danish Technical University in Lyngby for providing the control strains that were used to perform the PCRs and the technicians working at the VMDC of Utrecht University for collecting the isolates that met the inclusion criteria.

Funding

This work was supported by the Dutch Ministry of Economic Affairs, Agriculture and Innovation (grant number WOT-01-002-003).

Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://www.jac.oxfordjournals.org/).

References

1 Pfeifer Y, Cullik A, Witte W. Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. *Int J Med Microbiol* 2010; **300**: 371–9.

2 Hawkey PM, Jones AM. The changing epidemiology of resistance. *J Antimicrob Chemother* 2009; **64** Suppl 1: i3–10.

3 Pitout JD, Laupland KB. Extended-spectrum β-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *Lancet Infect Dis* 2008; **8**: 159–66.

4 Scientific Advisory Group on Antimicrobials of the Committee for Medicinal Products for Veterinary Use. Reflection paper on the use of third and fourth generation cephalosporins in food producing animals in the European Union: development of resistance and impact on human and animal health. *J Vet Pharmacol Ther* 2009; **32**: 515–33.

5 Dierikx C, van Essen-Zandbergen A, Veldman K *et al*. Increased detection of extended spectrum β-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. *Vet Microbiol* 2010; **145**: 273–8.

6 Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J *et al*. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin Microbiol Infect* 2011; **17**: 873–80.

7 Wieler LH, Ewers C, Guenther S *et al*. Methicillin-resistant staphylococci (MRS) and extended-spectrum β-lactamases (ESBL)-producing Enterobacteriaceae in companion animals: nosocomial infections as one reason for the rising prevalence of these potential zoonotic pathogens in clinical samples. *Int J Med Microbiol* 2011; **301**: 635–41.

8 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-first Informational Supplement M100-21*. CLSI, Wayne, PA, USA, 2011.

9 Wirth T, Falush D, Lan R *et al*. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol Microbiol* 2006; **60**: 1136–51.

10 Magiorakos AP, Srinivasan A, Carey RB *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2011; doi:10.1111/j.1469-0691.2011.03570.x.

11 Batchelor M, Hopkins KL, Liebana E *et al*. Development of a miniaturised microarray-based assay for the rapid identification of antimicrobial resistance genes in Gram-negative bacteria. *Int J Antimicrob Agents* 2008; **31**: 440–51.

12 Caroff N, Espaze E, Berard I *et al*. Mutations in the *ampC* promoter of *Escherichia coli* isolates resistant to oxyiminocephalosporins without extended spectrum β-lactamase production. *FEMS Microbiol Lett* 1999; **173**: 459–65.

13 Gay K, Robicsek A, Strahilevitz J *et al*. Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*. *Clin Infect Dis* 2006; **43**: 297–304.

14 Mulvey MR, Bryce E, Boyd DA *et al*. Molecular characterization of cefoxitin-resistant *Escherichia coli* from Canadian hospitals. *Antimicrob Agents Chemother* 2005; **49**: 358–65.

15 *SVARM 2009: Swedish Veterinary Antimicrobial Resistance Monitoring*. Uppsala, Sweden: National Veterinary Institute (SVA), 2010.

16 Costa D, Poeta P, Saenz Y *et al*. Prevalence of antimicrobial resistance and resistance genes in faecal *Escherichia coli* isolates recovered from healthy pets. *Vet Microbiol* 2008; **127**: 97–105.

17 Aarestrup FM, Hasman H, Veldman K *et al*. Evaluation of eight different cephalosporins for detection of cephalosporin resistance in *Salmonella enterica* and *Escherichia coli*. *Microb Drug Resist* 2010; **16**: 253–61.

18 Vo AT, van Duijkeren E, Fluit AC *et al*. Characteristics of extended-spectrum cephalosporin-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates from horses. *Vet Microbiol* 2007; **124**: 248–55.

19 Overdevest I, Willemsen I, Rijnsburger M *et al*. Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. *Emerg Infect Dis* 2011; **17**: 1216–22.

20 Paauw A, Fluit AC, Verhoef J *et al*. *Enterobacter cloacae* outbreak and emergence of quinolone resistance gene in Dutch hospital. *Emerg Infect Dis* 2006; **12**: 807–12.

21 Paauw A, Caspers MP, Leverstein-van Hall MA *et al*. Identification of resistance and virulence factors in an epidemic *Enterobacter hormaechei* outbreak strain. *Microbiology* 2009; **155**: 1478–88.

22 Leverstein-van Hall MA, Blok HE, Paauw A *et al*. Extensive hospital-wide spread of a multidrug-resistant *Enterobacter cloacae* clone, with late detection due to a variable antibiogram and frequent patient transfer. *J Clin Microbiol* 2006; **44**: 518–24.

23 *MARAN 2008: Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2008*. Lelystad, The Netherlands: Central Veterinary Institute (CVI) of Wageningen UR, 2010. www.cvi.wur.nl/NL/publicaties/rapporten/maranrapportage (17 February 2012, date last accessed).

24 Veldman K, Cavaco LM, Mevius D *et al*. International collaborative study on the occurrence of plasmid-mediated quinolone resistance in *Salmonella enterica* and *Escherichia coli* isolated from animals, humans, food and the environment in 13 European countries. *J Antimicrob Chemother* 2011; **66**: 1278–86.

25 Pomba C, da Fonseca JD, Baptista BC *et al*. Detection of the pandemic O25-ST131 human virulent *Escherichia coli* CTX-M-15-producing clone harboring the *qnrB2* and *aac(6′)-Ib-cr* genes in a dog. *Antimicrob Agents Chemother* 2009; **53**: 327–8.

26 Rogers BA, Sidjabat HE, Paterson DL. *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. *J Antimicrob Chemother* 2011; **66**: 1–14.

27 Ewers C, Grobbel M, Stamm I *et al*. Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-β-lactamase-producing *Escherichia coli* among companion animals. *J Antimicrob Chemother* 2010; **65**: 651–60.

28 Carattoli A, Garcia-Fernandez A, Varesi P *et al*. Molecular epidemiology of *Escherichia coli* producing extended-spectrum β-lactamases isolated in Rome, Italy. *J Clin Microbiol* 2008; **46**: 103–8.

29 Hasman H, Mevius D, Veldman K *et al*. β-Lactamases among extended-spectrum β-lactamase (ESBL)-resistant *Salmonella* from poultry, poultry products and human patients in The Netherlands. *J Antimicrob Chemother* 2005; **56**: 115–21.