Emergence and maintenance of multidrug-resistant *Escherichia coli* of canine origin harbouring a bla<sub>CMY-2</sub>-IncI1/ST65 plasmid and topoisomerase mutations

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Received 7 October 2013; returned 1 December 2013; revised 4 March 2014; accepted 9 March 2014

**Objectives:** To characterize the mechanisms implicated in fluoroquinolone (FQ) and expanded-spectrum cephalosporin (ESC) resistance in three clinical and seven faecal multidrug-resistant (MDR; resistant to at least three antimicrobial classes) *Escherichia coli* isolates from a dog with atopic dermatitis, also suffering from recurrent otitis, that had already been exposed to prolonged antimicrobial treatment and colonized for a long period.

**Methods:** MICs of FQs, ESCs and other antimicrobials were determined by the broth microdilution method. Phenotypic tests (efflux pump inhibition and combination disc tests) and isoelectric focusing were combined with genotypic analyses [PCRs, sequencing, conjugation, S1 nuclease PFGE, PCR-based replicon typing, plasmid multilocus sequence typing (pMLST) and PCR mapping] to characterize the molecular basis of FQ and ESC resistance. Isolates were further characterized by MLST and PFGE.

**Results:** Three otitis and five faecal isolates with enrofloxacin MICs of 32 to >128 mg/L displayed the GyrA:S83L+D87N/ParC:E62K/ParE:G545D pattern harbouring novel ParC and ParE substitutions, whereas the two remaining faecal isolates were susceptible or borderline resistant single-step mutants (GyrA:S83L pattern) and carried qnr<sub>S</sub>. Efflux pump overexpression also contributed to FQ resistance and the MDR phenotype. The three otitis and five faecal isolates also exhibited cefoxitin/ceftazidime MICs of 32–64 mg/L and harboured bla<sub>CMY-2</sub>, adjusted to ISeCp1, on an IncI1/ST65 conjugative plasmid, previously described in *Salmonella* Heidelberg from poultry. Interestingly, all isolates shared an identical MLST type (ST212), with the otitis isolates showing indistinguishable patterns with the high-level resistant faecal *E. coli* isolates.

**Conclusions:** The long-term maintenance of FQ- and ESC-resistant clones harbouring topoisomerase mutations and a bla<sub>CMY-2</sub>-IncI1/ST65 plasmid in canine commensal flora after prolonged antimicrobial use may contribute to the dissemination of multidrug resistance.

**Keywords:** DNA gyrase and topoisomerase IV mutations, AmpC, IncI1 plasmids, otitis, dogs

**Introduction**

The widespread use of antimicrobials in companion animals has recently been associated with the emergence of multiple resistance clones and/or determinants among pathogens and/or commensals.<sup>1,2</sup> *Escherichia coli* is commonly associated with canine urinary tract infections, bacterial dermatitis and otitis.<sup>1</sup> Despite recommendations to reserve broad-spectrum compounds, such as fluoroquinolones (FQs) and expanded-spectrum cephalosporins (ESCs), for only when susceptibility testing indicates that there are no alternatives, there is a tendency to use them as a first choice and/or empirically.<sup>1</sup> As a result, after the introduction of the first veterinary FQ, enrofloxacin, resistance mechanisms, mostly modifications in FQ target enzymes,
decreased FQ accumulation mainly through active efflux, and plasmid-mediated quinolone resistance (PMQR), have been identified among *E. coli* isolates from companion animals.1,3–6 Moreover, the steady increase in usage of β-lactamase inhibitors and first- to fourth-generation cephalosporins in companion animals has led to the emergence of ESC resistance in *E. coli* commonly related to the production of plasmid-mediated AmpC β-lactamases (pAmpCs) or extended-spectrum β-lactamases (ESBLs), thus raising a potential animal and public health concern.7,8

The objective of this study was to detect and characterize the mechanisms implicated in FQ and ESC resistance in clinical and faecal multidrug-resistant (MDR) *E. coli* isolates from a dog with recurrent otitis that had been rectally colonized for a long period.

Materials and methods

**Bacterial isolates**

Three *E. coli* isolates (E27OD, E34OD and E35OD) recovered from the ear exudates of anatomic dog with recurrent otitis externa and seven faecal isolates (K20FD and K201FD–K206FD) recovered from rectal swabs of the same animal were investigated. E27OD was recovered in July 2011 from the right external ear canal while the dog was on topical empirical treatment with human-labelled antimicrobials, initially with ciprofloxacin, which was then continued with the addition of ticarcillin/clavulanic acid. Three months later, bilateral otitis media was diagnosed and E34OD and E35OD were recovered (see Figure 1 and Figure S1) (available as Supplementary data at JAC Online). Six months before the diagnosis of otitis externa, the dog had been treated systemically with the human-labelled cefuroxime and with enrofloxacin for complicated bacterial dermatitis. The faecal isolates were recovered in a re-examination in May 2012, when ear exudates were negative for *E. coli* (see Figure 1 and Figure S1). Culture for faecal specimens was performed by selective enrichment initially into MacConkey broth and then onto MacConkey agar, both supplemented with amoxicillin (50 mg/mL; Sigma Chemical Co.); seven colonies were randomly selected. All isolates were identified by IMViC testing and use of the Vitek 2 system and were stored at –80°C.

**Susceptibility testing and screening for efflux pump overexpression and β-lactamase production**

MICs of selected antimicrobials for otitis and faecal *E. coli* isolates were determined by the broth microdilution method.8–10 ATCC 25922 was used as an *E. coli* control strain. MICs were also calculated in the presence of an efflux pump inhibitor (EPI; 80 mg/L).11 (see Table 1). Efflux pump overexpression was considered present when the MIC of enrofloxacin determined in medium containing EPI was decreased by a factor of ≥4. Screening for β-lactamase (pAmpCs and/or ESBLs) production was performed using the double disc synergy test, the boronic acid method and the three-dimensional test.7 β-Lactamase isoelectric points (pIs) were determined by analytical isoelectric focusing (IEF) using a β-lactamase mixture with known pIs [TEM-1 (5.4), PSE-2 (6.1), SHV-1 (7.6), SHV-5 (8.2) and LAT-1 (9.4)] as a control.

**Detection of FQ and ESC resistance mechanisms**

For the detection of mutations in genes encoding FQ target enzymes DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*), selected PCR and sequencing primers were used to amplify fragments including, but not limited to, the quinolone resistance-determining region (QRDR) (Table S1, available as Supplementary data at JAC Online). Sequence analysis and comparisons were conducted with MEGA5 software (http://www.megasoftware.net/). The presence of PMQR genes (*qnrA*, *qnrB*, *qnrS*, *aac(6’)-Ib-cr*, *qoxA*, *qoxB* and *qepA*) as well as bla genes encoding ESBLs (blaTEM, blaSHV and blaCTX-M) and six pAmpC groups were screened by previously described PCR assays; positive isolates were re-amplified using specific primers (Table S1).

**Conjugation experiments and plasmid analysis**

Transfer of ESC resistance was attempted by plate-mating with *E. coli* 26R793 (Lac+ Rif) as the recipient strain. Transconjugants were selected on MacConkey agar containing amoxicillin (50 mg/L) and rifampicin (200 mg/L). The detection of plasmids and the estimation of their size were carried out using S1 nuclease PFGE. Plasmids were typed by PCR-based replication typing13 and plasmid multilocus sequence typing (pMLST).14 Allele sequences were submitted to the http://pubmlst.org/plasmid/ web site. The bla*TEM* locus was determined by PCR mapping (Table S1).

**MLST and PFGE typing**

The genetic relatedness of isolates was assessed by MLST performed according to the *E. coli* web site protocol and alleles were submitted (http://mlst.ucc.ie/mlst/dbs/Ecoli/). PFGE of XbaI-digested genomic DNA was carried out according to CDC-pulseNet standardized procedure15 using the Fingerprinting II Software3 (Bio-Rad Laboratories).

**Nucleotide sequence accession numbers**

The nucleotide sequences from this study have been assigned to GenBank database accession numbers KC585021–KC585026 and JX997965–JX997972.

**Results and discussion**

**FQ resistance determinants**

All otitis and five faecal *E. coli* isolates exhibiting moderate to high-level resistance to the FQs tested (MIC 4 to >128 mg/L) presented a new GyrA:S83L+D87N/ParC:E62K/ParE:G545D amino acid substitution pattern harbouring two novel parC and parE mutations (Figure 1, Table 1 and Table S2). The predominance of GyrA substitutions in codons 83 and 87 found among the FQs tested (Figure1, Table 1 and Tables S2 to S4) (available as Supplementary data at JAC Online). The remaining two faecal isolates presented the GyrA:S83L pattern with one of them, K20FD, exhibiting susceptibility (MIC 0.06–0.125 mg/L) and the other, K206FD, exhibiting borderline resistance (MIC 2–4 mg/L) to the FQs tested (Figure 1, Table 1 and Table S2). The predominance of GyrA substitutions in codons 83 and 87 found among FQ-resistant *E. coli* from companion animals1–6,12,13 indicates a key role in conferring high-level FQ resistance, possibly involving a loss of the ability to form hydrogen bonds or the loss of a negatively charged amino acid at these positions.16 The novel substitution E62K involves the transition to a positive charge (E+ to K+). However, the exact role and contribution to FQ resistance of similar substitutions, such as E84K also reported in high-level resistant *E. coli* from companion animals,17 needs investigation.

Isolates with the same topoisomerase substitutions exhibited differing MICs for FQs (Table 1), indicating that additional mechanisms contributed to overall resistance.5,6 Indeed, efflux pump activity was confirmed since, using enrofloxacin as standard, all *E. coli* isolates showed MIC reductions by a factor of 4 to >32 (Table 1). Nevertheless, the MIC reduction varied depending on the FQ molecule: the magnitude of the decrease was greatest for enrofloxacin, followed by marbofloxacin, pradofloxacin and...
Figure 1. Characteristics and resistance mechanisms of E. coli isolates analysed in this study. The dendrogram shows comparisons of PFGE patterns for the three otitis (†) and seven faecal (*) canine E. coli isolates based on the unweighted pair-group with arithmetic averages clustering method; percentage similarities are shown above the dendrogram. Dashes indicate the absence of corresponding genetic characteristics.

Table 1. MICs of β-lactams, quinolones and other antimicrobial agents for otitis and faecal E. coli isolates, their transconjugants and the E. coli recipient

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<th>CXM</th>
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Transconjugants carrying bla<sub>CMY</sub>-2

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Recipient strain

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AMP, ampicillin; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CXM, cefuroxime; FOX, cefoxitin; CAZ, cefazidime; FEP, cefepime; NAL, nalidixic acid; ENR, enrofloxacin; CIP, ciprofloxacin; MAR, marbofloxacin; PRA, pradofloxacin; DOX, doxycycline; TMP, trimethoprim; SFD, sulfadiazine; ND, not determined.

MICs were determined in duplicate and interpreted according to CLSI criteria; for pradofloxacin, the MIC breakpoints proposed by the manufacturer and presented by the European Medicines Agency were used (EMEA/CVMP/342257/2007). MICs calculated in the absence and presence of an EPI, Phe-Arg<sup>-</sup>-naphthylamide (PAPN; Sigma Chemical Co.), are shown; the magnitude of the MIC decrease in the presence of the EPI is presented in brackets.
ciprofloxacin (Table 1), probably reflecting the different lipophilicity of these FQs.17,18 Furthermore, the presence of the EPI increased the susceptibility of all isolates to doxycycline (Table 1), indicating a possible role of the efflux-mediated FQ resistance in acquiring cross-resistance to structurally unrelated antimicrobials and the MDR phenotype.3,12

Regarding PMQR, the qnrS1 gene was found only in the two faecal E. coli isolates exhibiting FQ susceptibility or borderline resistance. Consequently, the presence of qnrS1 probably had a minimal contribution to FQ resistance, especially since these isolates also exhibited GyrA substitution and efflux pump activity (Figure 1 and Table 1). However, the spread of PMQR genes can serve as a potential reservoir in the formation of the MDR phenotype,4 possibly facilitating the selection of FQ-resistant strains.

ESC resistance determinants

The otitis and faecal isolates that were fully resistant to FQs also displayed cefoxitin/ceftazidime resistance, but no resistance to cefepime (Table 1), suggesting the possibility of AmpC β-lactamase production, which was confirmed by the brononic acid method, the three-dimensional test and IEF (pf 9). PCRs and sequencing confirmed the presence of bla_{CMY-2} and bla_{TEM-1} in the above isolates. The remaining two faecal E. coli isolates carried only bla_{TEM-1}, exhibiting MICs that were decreased 4- to 10-fold compared with the otitis and faecal AmpC-producers. Notably, the emergence of bla_{CMY-2} positive E. coli in canine otitis, in contrast to other clinical cases, is globally rare.9

The cefoxitin/ceftazidime resistance phenotype was transferred by conjugation for all bla_{CMY-2} positive E. coli (frequency 0.6–1 × 10^{-9}danor), with the respective transconjugants acquiring bla_{CMY-2}, but not bla_{TEM-1}, and presenting an 11- to 12-fold increase in cefoxitin MIC compared with the recipient (Table 1). Interestingly, the resistance to doxycycline, trimethoprim and sulfadiazine was not transferred. S1 PFGE revealed that only one large plasmid (about 66 kbp) from the two or three plasmids shared among the otitis E. coli isolates was transferable (Figure S2, available as Supplementary data at JAC Online). All conjugative plasmids were assigned to the I1 incompatibility group (IncI1) (Figure 1). Remarkably, bla_{CMY-2}-IncI1 plasmids belonged to ST65 (allelic profile, araA4, pilI3, repI1, sog5:10, trbA:18), previously identified in Salmonella Heidelberg from poultry, and not to ST2, which has so far only been detected in canine E. coli; notably, IncI1/ST2 is also a common human plasmid type (Table S5, available as Supplementary data at JAC Online). Meanwhile, an IS2911 element was identified 116 bp upstream of bla_{CMY-2}, suggesting a capability for mobilization and expression.7,16

**Emergence and maintenance of MDR E. coli**

Canine otitis is a common and multifactorial cutaneous disorder, in which opportunistic infections are a major reason for treatment failure. Empirical topical antimicrobial administration is the recommended initial treatment for canine otitis externa, since high concentrations are achieved within the ear canal.19 However, when an isolate already exhibiting high-level FQ resistance is found in the ear canal, therapeutic failure can occur.12 In our case, the prolonged systemic administration of enrofloxacin during the pre-admission period (Figure S1) resulted in the emergence of resistant E. coli and intestinal colonization with it. Two clonally related (ST212) E. coli subpopulations were obtained from faecal microflora, the resistant one harbouring multiple mutations (PFGE type A) and the more susceptible single-step mutant (PFGE type B), with the former being predominant and involved in otitis (92.9%–100% PFGE pattern identity with otitis isolates) (Figure 1). Besides this, the continuous emergence of FQ resistance involving additive mechanisms, e.g. efflux pump activity, seems to have occurred within the subpopulations (Table 1). Indeed, in vivo studies in dogs have shown that FQ administration impacts the composition of the faecal E. coli population in favour of MDR strains.3,20 High carriage of E. coli resistant towards ESCs also has been shown after β-lactam administration.3,20,21 Prolonged administration of cefuroxime, in our case (Figure S1), may be linked to the selection of E. coli producing IncI1/ST65-CMY-2 β-lactamase. A bla_{CMY-2}-IncI1/ST65 plasmid has been detected in Salmonella Heidelberg from chicken breast in the USA.22 The possible transfer of bla_{CMY-2}-IncI1 plasmids sharing an identical ST between commensal E. coli and Salmonella serotypes and across hosts and continents has previously been suggested (Table S5). Taking into account that IncI1 prevalence seems to be linked to a particular reservoir of E. coli and Salmonella from poultry14 and that Salmonella isolates exhibiting an ESC-resistant phenotype have been found in poultry-derived products in our country (i.e. Greece),23 we can assume that, in our dog, the bla_{CMY-2}-IncI1/ST65 plasmid might have been transferred from a poultry clone. The question now raised is how the E. coli bearing the bla_{CMY-2}-IncI1/ST65 plasmid was maintained for more than 1 year (Figure S1), a period apparently free from systemic administration. The maintenance of a bla_{CMY-2}-IncI1 plasmid under non-selective conditions could have resulted from the reduced biological cost achieved during the initial selection process.24

**Conclusions**

To our knowledge, this is the first report of a bla_{CMY-2}-IncI1/ST65 conjugative plasmid in E. coli from companion animals also expressing the MDR phenotype associated with FQ resistance. The long-term maintenance of FQ and ESC resistance clones and determinants in canine commensal flora after prolonged antimicrobial use might create a potential resistance reservoir. Good treatment practices could restrict the selection of resistant canine E. coli and improve the therapeutic outcome using the currently registered as well as next-generation veterinary compounds.

**Acknowledgements**

We are very grateful to Professor George A. Jacoby (Lahey Clinic Medical Center, Burlington, MA, USA) for kindly providing E. coli strains positive for qnr (J53 pMG252, qnrA1; J53 pMG298, qnrB1; and J53 pMG306, qnrS1), aac(6’)-Ib-cr (J53 pMG298) and aepA (J53 pAT851), to Dr Alessandra Carattoli (Research Director at Istituto Superiore di Sanita’, Italy) for the generous supply of detailed and updated information from pMLST web site data and to Dr Jason Folster (CDC, Atlanta, GA, USA) for sharing aspects of his research on IncI1 plasmids. We are also grateful to Georgia Kythreotou and Theofilos Papadopoulos for their assistance. We would also like to thank Bayer HealthCare AG, Germany, for kindly supplying ciprofloxacin, enrofloxacin and pradofloxacin, and Vetoquinol S A, France, for kindly providing marbofloxacin.
Funding
This study was supported by internal funding and by a proportion of E. I. V.’s scholarship from the Greek State Scholarships Foundation (I.K.Y.).

Transparency declarations
None to declare.

Supplementary data
Figure S1, Figure S2 and Tables S1 to S5 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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