Antimicrobial-resistant *Escherichia coli* in hospitalised companion animals and their hospital environment

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INTRODUCTION: Antimicrobial resistance is a growing concern with implications for animal health. This study investigated the prevalence of antimicrobial resistance among commensal and environmental *Escherichia coli* isolated from animals sampled in referral hospitals in the UK.

MATERIALS AND METHODS: Resistant *Escherichia coli* isolated from animal faeces and practice environments were tested for susceptibility to antimicrobial agents. PCR and sequencing techniques were used to identify extended spectrum beta-lactamase and AmpC-producer genotypes.

RESULTS: In total, 333 faecal and 257 environmental samples were collected. Multi-drug resistant *Escherichia coli* were found in 13·1% of faecal and 8·9% of environmental samples. Extended spectrum beta-lactamase and AmpC genes were identified 14% and 7·7% of faecal samples and 8·6% and 8·6% of environmental samples, respectively. The most common extended spectrum beta-lactamase gene type detected was $bla_{CTX-M\ -15}$, although $bla_{TEM-158}$ was detected in faecal and environmental samples from one practice.

CLINICAL SIGNIFICANCE: *Escherichia coli* resistant to key antimicrobials were isolated from hospitalised animals and the practice environment. We identified the emergence of the inhibitor resistant and extended spectrum beta-lactamase $bla_{TEM-158}$ in companion animals. Further investigation to determine risk factors for colonisation with antimicrobial-resistant bacteria is needed to provide evidence for antimicrobial stewardship and infection control programmes.

INTRODUCTION

Antimicrobial resistance has the potential for major impact on animal welfare, limiting therapeutic options. Antimicrobial resistant (AMR) infections can increase morbidity and mortality and, furthermore, have been shown to be associated with an increased financial burden in human (Baker *et al.* 2012, Smith & Coast 2013, Tansarli *et al.* 2013) and veterinary (Dallap Schaer *et al.* 2010) medicine.

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Humans, dogs and other mammals carry commensal *Escherichia coli* (*E. coli*) within the gut where exposure to antimicrobial agents can select for resistance. Antimicrobial resistance in *E. coli* isolates from companion animals has been reported in both healthy dogs in the community (Carattoli *et al.* 2005, Sun *et al.* 2010, Wedley *et al.* 2011, Franiek *et al.* 2012, Tamang *et al.* 2012, Ben Sallem *et al.* 2013, Gandolfi-Decristophoris *et al.* 2013) and hospitalised animals (So *et al.* 2012). However, AMR burdens may be higher in hospitalised animals (Nam *et al.* 2010) and in animals undergoing treatment (Sun *et al.* 2010) compared to healthy animals. One AMR mechanism of importance among *E. coli* is the production of beta-lactamases (Sykes & Matthew 1976). Use of third and fourth generation cephalosporins has

contributed to the emergence of extended spectrum beta-lactamase (ESBL) enzymes (Pfeifer *et al.* 2010), which confer resistance to all beta-lactam antibiotics, although they remain sensitive to beta-lactamase inhibitors, such as clavulanic acid.

E. coli can also gain resistance to beta-lactam agents by the production of cephamycinase AmpC enzymes which, unlike ESBLs, also confer resistance to beta-lactamase inhibitors. In addition, ESBL genes frequently co-exist with other resistance genes on the same plasmid, resulting in resistance to multiple antimicrobial classes (Woodford *et al.* 2004, Pitout *et al.* 2007, Pitout & Laupland 2008, Schultsz & Geerlings 2012). This adds to the resistance burden within bacterial populations, because the use of one antimicrobial class could select for resistance to multiple other classes by favouring the spread of plasmids that confer multi-drug resistance (MDR – resistance to ≥three drug classes).

Antimicrobial-resistant *E. coli* have been isolated from faeces of animals in many parts of the world. Moreover, ESBL-producing bacteria have been associated with urinary tract infections (O'Keefe *et al.* 2010, Shaheen *et al.* 2011, Dierikx *et al.* 2012, Huber *et al.* 2013, Nebbia *et al.* 2014), wound infections (Steen & Webb 2007) and cholangiohepatitis in companion animals (Steen & Webb 2007, Timofte *et al.* 2011). Among ESBL-producing isolates from studies of companion animals the most common types of ESBLs identified have been CTX-M types (Carattoli *et al.* 2005, Sun *et al.* 2010, Dierikx *et al.* 2012, Tamang *et al.* 2012, Ben Sallem *et al.* 2013, Huber *et al.* 2013). However, data on the frequency and epidemiology of these bacteria within veterinary environments in the UK are lacking.

Therefore, the aim of this study was to determine the prevalence of important AMR *E. coli* phenotypes and genotypes in the faecal flora of hospitalised animals and the hospital environment in referral practices in the UK. This included resistance to antimicrobials critically important to human health (WHO 2011), such as the third and fourth generation cephalosporins and fluoroquinolones, which are also important in companion animals, as well as resistance to commonly used antimicrobials in companion animals, such as clavulanic acid-potentiated amoxicillin and potentiated sulphonamides. In addition, we aimed to characterise and compare the genes associated with ESBL-producing and AmpC-producing *E. coli* in the environment and shed in faeces.

MATERIALS AND METHODS

Practice selection and sample collection

Five referral hospitals in Northwest UK took part in the study. The main inclusion criterion was that the hospital must accept secondary referral patients, including cases likely to be hospitalised. The limitation to the Northwest region was to facilitate the sample collection and minimise time between collection and laboratory processing. Written informed consent was obtained from all owners whose animals took part in the study. Ethical approval was granted by the University Research Ethics Committee. Sample size estimates indicated that with an expected prevalence of ESBL-producing *E. coli* of 10%, 385 faecal samples would be required to determine the prevalence with a precision of 3 and 95% confidence. Hence the aim was to collect 385 animal faecal samples in addition to the environmental samples.

Animals eligible for the study were all dogs and cats hospitalised for at least one night in the practice. Day cases were excluded as were animals receiving chemotherapy or radiotherapy and those in isolation. The target for sample collection was one faecal sample per hospitalised animal per day. Samples were collected by practice staff, stored in cool-boxes at the practice and collected at regular intervals.

Sampling was rotated and performed in three blocks of 2 weeks at each practice apart from one practice that included an extra pilot week. Environmental samples were collected by the author weekly once during each week of sampling from each practice. There was some variation in the exact sites selected for weekly sampling in each practice necessitated by the different layouts. However, the areas sampled from all practices were: ward floors; computer keyboards in kennel rooms and treatment areas; examination tables in treatment areas (not in consulting rooms); and the outside dog walking areas. If there were multiple keyboards or examination tables in the same area, one was selected on the first week of sampling and was used for all subsequent sample collection. Samples from floors were collected by wearing sterile disposable absorbent overshoes (boot socks) moistened with 2 to 3 mL sterile saline, walked around rooms in the same pattern each week. Samples were taken from keyboards and tables using sterile j-clothes that are cut to a standard size ($\sim 15\,cm^2$), moistened with sterile saline and used to wipe over the entire surface of keyboards and tables.

Isolation of resistant bacteria from faecal and environmental samples

Samples were transported to the laboratory of University of Liverpool and processed immediately after collection. For faeces, 2 g was homogenised in brain heart infusion broth and used to inoculate the entire surface of an eosin methylene blue agar plate (EMBA). Antibiotic discs impregnated with ampicillin (10 μg), clavulanic acid potentiated amoxicillin (Augmentin®; potentiated amoxicillin, 30 μg), ciprofloxacin (1 μg) and trimethoprim (2·5 μg) were added to the plate, which was then incubated overnight at 37°C (Bartoloni *et al.* 2006). A 0·5-mL aliquot of the faecal homogenate was added to 4·5-mL buffered peptone water and incubated over night at 37°C before processing as below.

Boot socks and cloths were agitated in 20-mL buffered peptone water for 2 minutes and a 10-mL aliquot incubated overnight at 37°C. After overnight incubation environmental samples were processed as below.

The faecal and environmental homogenates after overnight enrichment were streaked onto 1 EMBA plate containing 1 μg/mL cefotaxime and another containing 1 μg/mL ceftazidime to isolate cephalosporin-resistant *E. coli*, and incubated overnight at 37°C.

A maximum of six potential AMR *E. coli* isolates could be selected per faecal sample if present: one each from the four inhibition zones on the plate containing antimicrobial discs, one from each of the cephalosporin-impregnated EMBA plates. Each environmental sample yielded a maximum of two isolates if typical growth was present on the cephalosporin-impregnated plates. All isolates were then subjected to full antimicrobial susceptibility testing as detailed below.

Antimicrobial susceptibility and ESBL phenotypic testing

Full susceptibility testing was performed on all isolates using iso-sensitest agar (LabM, UK) with discs impregnated with 10-μg ampicillin, 30-μg potentiated amoxicillin, 30-μg chloramphenicol, 30-μg nalidixic acid, 1-μg ciprofloxacin, 2·5-μg trimethoprim and 30-μg tetracycline, according to BSAC guidelines (BSAC 2013).

Isolates from the ceftazidime and cefotaxime EMBA plates, or which were resistant to CAPA, were also tested for ESBL phenotypes and AmpC phenotypes using the Mast Ltd (Bootle, UK) double disc set according to the manufacturer's instructions (M'Zali *et al.* 2000), which has been found to have 90% sensitivity and specificity for the detection of AmpC-producer phenotypes (Ingram *et al.* 2011).

All antibiotic discs were obtained from Mast Ltd, the antibiotic powders from Sigma–Aldrich UK and all media from Lab M Ltd (Bury, UK).

Genotypic analysis

Cell lysates were prepared by adding two to three colonies of pure 24-hour cultures to 0·5 mL sterile water and heating at 100°C for 20 minutes. All isolates morphologically consistent with *E. coli* were confirmed using *uidA* gene primers (McDaniels *et al.* 1996, Maddox *et al.* 2011). All isolates demonstrating resistance to cefotaxime and ceftazidime on sensitivity testing were tested for bla_{CTX-M} genes using universal bla_{CTX-M} primers as previously described (Batchelor *et al.* 2005). To determine the CTX-M group produced, all CTX-M positive isolates were tested using primers specific to bla_{CTX-M} groups 1, 2(Hopkins *et al.* 2006) and 9 (Batchelor *et al.* 2005). All isolates identified as producing CTX-M group 1 ESBLs were tested to further determine whether they belonged to serogroup O25 (Clermont *et al.* 2008) and for markers for sequence type (ST) 131 (Clermont *et al.* 2009), representing the pandemic *E. coli* clone associated with $bla_{CTX-M-15 in}$ human clinical infections (Lau *et al.* 2008)

All isolates demonstrating ceftazidime resistance on sensitivity testing were also tested for bla_{TEM}, bla_{SHV} and bla_{OXA} genes using a further multiplex PCR assay (Dallenne *et al.* 2010). All isolates demonstrating potentiated amoxicillin resistance on sensitivity testing were tested for bla_{CITM} using CITM primers (Perez-Perez & Hanson 2002). The specific genotypes of beta-lactamase genes (bla_{CTX-M}, bla_{TEM}, bla_{CMY}) were further determined with gene amplicons sequenced (Perez-Perez & Hanson 2002, Boyd *et al.* 2004, Batchelor *et al.* 2005) at the Zoology Sequencing Facility, University of Oxford. The specific genes were determined by comparing the sequences with those submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank).

All isolates demonstrating quinolone resistance were tested for the presence of the plasmid-mediated quinolone resistance genes, *qnrA*, *qnrB* and *qnrS* using a multiplex PCR assay as previously described (Robicsek *et al.* 2006).

Statistical analysis

Descriptive statistics and 95% confidence intervals were calculated for overall prevalence and for each practice. Resistance to each antimicrobial was considered as a separate outcome. The other outcomes considered were: MDR *E. coli*; resistance to third generation cephalosporins; and ESBL- and AmpC-producer phenotypes and genotypes.

Data were clustered within veterinary practices and, because of repeated sampling, also within animal, so to obtain accurate estimates after allowing for this clustering the prevalence of AMR *E. coli* was estimated using multilevel, binomial models for each outcome, with practice and dog clustering accounted for by incorporation of second- and third-level random intercept terms. Calculations were performed using penalised quasi-likelihood estimates (second- order or first-order PQL). The prevalence (P_T) was estimated using the formula below, incorporating the constant parameter estimate (β_0) derived from the random intercept-only 3-level models for each outcome considered:

$$P_T = e^{\beta_0}/1 + e^{\beta_0}$$

95% confidence intervals for all adjusted prevalence estimates were constructed by examination of the standard errors of the intercept-only model parameters. Data were analysed using the MLwiN statistical software package (MLwiN Version 2.1 Centre for Multilevel Modelling, University of Bristol, UK).

RESULTS

A total of 333 faecal samples were collected from 214 animals over 31 sampling weeks between May 13 and October 21, 2013 (Table 1). In total 348/363 faecal isolates and 77/86

Table 1. Total number of faecal and environmental samples collected for each practice. Faecal samples are categorised by species and environmental samples by location

Practice	Faecal samples*				Environmental samples†				
	Dogs	Cats	Not specified	Total	Inside floors	Examination tables	Keyboards	Outdoors walking area	Total
1	122	14	0	136	28	12	18	7	65
2	32	0	5	37	30	6	6	6	48
3	63	10	0	73	24	6	12	6	48
4	34	0	2	36	24	6	12	6	48
5	45	0	6	51	30	6	6	6	48
Total	296	24	13	333	136	36	54	31	257

*This included repeated samples from animals. The median number of samples per animal was 2 (interquartile range 1 to 3) and ranged from a minimum of 1 to a maximum of 14
†Environmental samples were collected by the author once weekly during each week of sampling, and the sites sampled were selected at the beginning of the study and remained constant. All hospitals were sampled for 6 weeks except for hospital 1 which had an additional pilot week

environmental isolates tested positive using the *uidA* PCR test confirming the *E. coli* identity of these isolates.

E. coli resistant to at least one antimicrobial were isolated from 167/333 (50·1%) faecal samples. A total of 257 environmental samples were collected during the same 31 sampling weeks (Table 1). Of these, 47 (18·3%) contained AMR *E. coli* cultured from cephalosporin selective plates.

The overall prevalence of faecal and environmental samples with at least one isolate with resistance to one or more of the six antimicrobials, to third generation cephalosporins, MDR, and ESBL-producer and AmpC-producer phenotypes, and ESBL and bla_{CITM} genotpyes is shown in Tables 2 and 3. Ampicillin resistance was the most common resistance type in both faecal and environmental samples.

There was considerable variation in the rate of isolation of resistant bacteria between practices and between areas within practices (Fig 1, and Tables 2 and 3). Practice 1 demonstrated the highest prevalence of potentiated amoxicillin resistance, ciprofloxacin resistance, and AmpC-phenotype and genotype [bla_{AmpC} (CITM)] in both faecal and environmental samples. Practice 3 demonstrated the highest prevalence of ESBL-phenotype and genotype, and MDR *E.coli* among samples. Practices 2 and 5 generally demonstrated lower levels of resistance. The resistance spectrum varied among the MDR isolates from both faecal and environmental samples (Table 4). There were 18 distinct phenotypes among MDR isolates, 17 in the faecal samples and 10 in the environmental samples (although there was substantial overlap). The diversity of *E. coli* MDR phenotypes from environmental samples may have been limited due to isolation only on cephalosporin-containing media following enrichment with only two possible colony picks per sample, unlike faecal samples where up to six colony picks could be obtained. Many MDR isolates were resistant to ciprofloxacin and potentiated amoxicillin. Environmental samples associated with ESBL-, AmpC-producing and MDR *E. coli* were most likely to be isolated from either the outside walking areas, or floors of the ward communal areas (Fig 1).

PCR detected bla_{CTX-M} genes belonging to group 1 in *E. coli* from 22 (6·6%) faecal samples from four out of five practices and 12 (4·7%) environmental samples from three out of five practices. Group 9 bla_{CTX-M} genes were detected in *E. coli* from 3 (0·9%) faecal samples and 2 (0·8%) environmental samples, all of which were from the same practice. No isolates were positive for CTX-M group 2, or found to belong to O25/ST131 clones by PCR assay.

The bla_{CITM} gene was detected in *E. coli* from 72 (21·6%) faecal and 22 (8·6%) environmental samples, all of which were shown by sequence analysis to correspond to bla_{cmy-2}. This corresponded to 81% and 69% of faecal and environmental samples with *E. coli* with potentiated amoxicillin resistance, respectively. There was significant masking of ESBL producer phenotype by the production of AmpC with *E. coli* from 20 faecal samples and 2 environmental samples that had both AmpC and ESBL genes.

A low prevalence of *qnr* genes was found only in three (1%) faecal samples having at least one *E. coli* isolate which tested

Table 2. Sample level prevalence of resistance to each antimicrobial class tested in faecal samples (n=333) from each practice with 95% confidence intervals

Resistance phenotype	Practice 1	Practice 2	Practice 3	Practice 4	Practice 5	All practices (N)	All Practices	All practices adjusted for clustering*
Ampicillin	58·1 (49·8 to 66·4)	37·8 (22·2 to 53·5)	49·3 (37·8 to 60·8)	41·7 (25·6 to 57·8)	39·2 (25·8 to 52·6)	164	49·2 (43·9 to 54·6)	45·4 (36·6 to 54·6)
CAPA	42·6 (34·3 to 51)	24·3 (10·5 to 38·1)	20·5 (11·3 to 29·8)	19·4 (6·5 to 32·4)	15·7 (5·7 to 25·7)	97	29·1 (24·2 to 34)	14·0 (6·7 to 27·0)
Chloramphenicol	19·9 (13·1 to 26·6)	24·3 (10·5 to 38·1)	24·7 (14·8 to 24·5)	2·8 (0 to 8·1)	5·9 (0 to 12·3)	58	17·4 (13·3 to 21·5)	13·5 (8·7, 20·4)†
Tetracycline	12·5 (6·9 to 18·1)	5·4 (0 to 12·7)	15·1 (6·9 to 23·3)	11·1 (0·8 to 21·4)	19·6 (8·7 to 30·5)	44	13·2 (9·6 to 16·9)	12·8 (9·5, 17·0)
Trimethoprim	32·4 (24·5 to 40·2)	24·3 (10·5 to 38·1)	30·1 (19·6 to 40·7)	13·9 (2·6 to 25·2)	13·7 (4·3 to 23·2)	87	26·1 (21·4 to 30·8)	12·7 (6·8, 22·4)
Ciprofloxacin	44·1 (35·8 to 52·5)	8·1 (0 to 16·9)	11·0 (3·8 to 18·1)	13·9 (2·6 to 25·2)	7·8 (0·5 to 15·2)	80	24·0 (19·4 to 28·6)	9·2 (3·2, 23·9)
MDR	31·6 (23·8 to 39·4)	24·3 (10·5 to 38·1)	34·2 (23·4 to 45·1)	22·2 (8·6 to 35·8)	9·8 (1·6 to 18·0)	115	27·0 (22·3 to 31·8)	13·1 (6·9 to 23·6)
Third generation cephalosporin	50·0 (41·6 to 58·4)	24·3 (10·5 to 38·1)	41·1 (29·8 to 52·4)	36·1 (20·4 to 51·8)	11·8 (2·9 to 20·6)	126	37·8 (32·6 to 43)	27·2 (14·9 to 44·3)
ESBL-producer phenotype	14·0 (8·1 to 19·8)	8·1 (0 to 16·9)	30·1 (19·6 to 40·7)	25·0 (10·9 to 39·1)	0·0	53	15·9 (12 to 19·8)	9·7 (3·4 to 24·6)
AmpC-producer phenotype	33·1 (25·2 to 41)	0·0	1·4 (0 to 4)	5·6 (0 to 13)	2·0 (0 to 5·8)	49	14·7 (10·9 to 18·5)	4·3 (1·1 to 15·6)†
ESBL-production confirmed‡	26·5 (19·1 to 33·9)	8·1 (0 to 16·9)	34·2 (23·4 to 45·1)	22·2 (8·6 to 35·8)	2·0 (0 to 5·8)	73	21·9 (17·5 to 26·4)	14·0 (5·3, 35·0)
bla_{CITM} positive PCR	38·2 (30·1 to 46·8)	13·5 (2·5 to 24·5)	11·0 (3·8, 18·1)	13·9 (2·6, 25·2)	3·9 (0 to 9·2)	72	21·6 (17·2 to 26)	7·7 (2·5, 21·1)

MDR multidrug resistance, CAPA clavulanic acid potentiated amoxicillin, ESBL Extended spectrum beta-lactamase
*Estimates from intercept only multilevel models adjusted for both repeated measures within dog and clustering within practice and hence represents the average sample level prevalence adjusted for repeated samples within dog and for clustering within hospitals
†Estimates from first order penalised quasi-likelihood
‡Confirmed ESBL producer if either had an ESBL producer phenotype, was positive on the universal bla_{CTXM} PCR or, in the case of TEM and SHV producer types, returned a sequencing result corresponding to an ESBL.

Table 3. Sample level prevalence of resistance to each antimicrobial class tested in environmental isolates from cephalosporin-impregnated media (n=257) from each practice with 95% confidence intervals

Resistance phenotype	Practice 1	Practice 2	Practice 3	Practice 4	Practice 5	All practices (N)	Total
Ampicillin	33·8 (22·3 to 45·3)	6·3 (0 to 13·1)	25·0 (12·8 to 37·3)	12·5 (3·1 to 21·9)	6·3 (0 to 13·1)	46	17·9 (13·2 to 22·6)
CAPA	29·2 (18·2 to 40·3)	6·3 (0 to 13·1)	8·3 (0·5 to 16·2)	4·2 (0 to 9·8)	2·1 (0 to 6·1)	29	11·3 (7·4 to 15·2)
Chloramphenicol	6·2 (0·3 to 12)	0·0	14·6 (4·6 to 24·6)	4·2 (0 to 9·8)	0·0	13	5·1 (2·4 to 7·7)
Tetracycline	6·2 (0·3 to 12)	6·3 (0 to 13·1)	6·3 (0 to 13·1)	4·2 (0 to 9·8)	4·2 (0 to 9·8)	14	5·4 (2·7 to 8·2)
Trimethoprim	12·3 (4·3 to 20·3)	4·2 (0 to 9·8)	18·8 (7·7 to 29·8)	2·1 (0 to 6·1)	2·1 (0 to 6·1)	23	8·9 (5·5 to 12·4)
Ciprofloxacin	29·2 (18·2 to 40·3)	2·1 (0 to 6·1)	6·3 (0 to 13·1)	4·2 (0 to 9·8)	2·1 (0 to 6·1)	27	10·5 (6·8 to 14·3)
MDR	15·4 (6·6 to 24·2)	4·2 (0 to 9·8)	14·6 (4·6 to 24·6)	4·2 (0 to 9·8)	4·2 (0 to 9·8)	23	8·9 (5·5 to 12·4)
Third generation cephalosporin	33·8 (22·3 to 45·3)	2·1 (0 to 6·1)	22·9 (11 to 34·8)	8·3 (0·5 to 16·2)	6·3 (0 to 13·1)	41	16·0 (11·5 to 20·4)
ESBL-producer phenotype	3·1 (0 to 7·3)	0·0	16·7 (6·1 to 27·2)	8·3 (0·5 to 16·2)	0·0	14	5·4 (2·7 to 8·2)
AmpC-producer phenotype	16·9 (7·8 to 26)	0·0	2·1 (0 to 6·1)	0·0	0·0	12	4·7 (2·1 to 7·2)
ESBL confirmed*	13·8 (5·4 to 22·2)	0·0	16·7 (6·1 to 27·2)	10·4 (1·8 to 19·1)	0·0	22	8·6 (5·1 to 12)
bla$_{CTM}$ positive PCR	23·1 (12·8 to 33·3)	2·1(0 to 6·1)	4·2 (0 to 9·8)	6·3 (0 to 13·1)	2·1 (0 to 6·1)	22	8·6 (5·1 to 12)

MDR multidrug resistance, CAPA clavulanic acid potentiated amoxicillin, ESBL - Extended spectrum beta-lactamase
*Confirmed ESBL producer if either had an ESBL-producer phenotype, was positive on the universal bla$_{CTXM}$ PCR or, in the case of TEM and SHV producer types, returned a sequencing result corresponding to an ESBL

Table 4. The different antimicrobial resistance profiles seen among the multi-drug resistance phenotypes in both faecal and environmental samples

Resistance phenotype	Number of antimicrobial classes resistant to	Faecal samples		Environmental samples	
		Number of samples	% (95% CI)	Number of samples	% (95% CI)
amp aug chl trim nal cip	4	20	23·3 (14·3 to 13·2)	2	8·7 (0 to 20·2)
amp chl trim	3	13	15·1 (7·5 to 22·7)	4	17·4 (1·9 to 32·9)
amp aug chl tet trim nal cip	5	8	9·3 (3·2 to 15·4)	4	10·5 (0·8 to 20·3)
amp aug trim nal cip	3	7	8·1 (2·4 to 13·9)	3	13 (0 to 26·8)
amp aug chl trim	3	6	7·0 (1·6 to 12·4)	1	4·3 (0 to 12·7)
amp tet trim nal cip	4	5	5·8 (0·9 to 10·8)	2	8·7 (0 to 20·2)
amp chl tet trim nal cip	5	5	5·8 (0·9 to 10·8)		
amp tet nal	3	4	4·7 (0·2 to 9·1)		
amp aug tet trim nal cip	4	4	4·7 (0·2 to 9·1)	1	4·3 (0 to 12·7)
amp tet trim	3	3	3·5 (0 to 7·4)		
amp aug tet trim	3	3	3·5 (0 to 7·4)	2	8·7 (0 to 20·2)
amp chl trim nal cip	4	2	2·3 (0 to 5·5)		
amp trim nal cip	3	1	1·2 (0 to 3·4)		
amp tet nal cip	3	1	1·2 (0 to 3·4)	2	8·7 (0 to 20·2)
amp chl tet trim nal	5	1	1·2 (0 to 3·4)		
amp aug chl tet trim	4	1	1·2 (0 to 3·4)		
amp aug chl nal cip	3	1	1·2 (0 to 3·4)		
amp chl tet trim	4			2	8·7 (0 to 20·2)

amp ampicillin resistant, aug CAPA resistant, chl chloramphenicol resistant, tet tetracycline resistant, trim trimethoprim resistant, nal nalidixic acid resistant, cip ciprofloxacin resistant
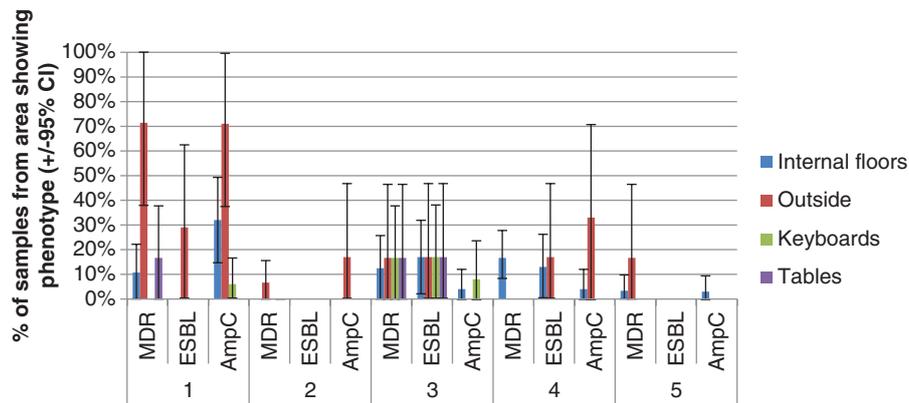
FIG 1. The percentage of samples from each area within each practice yielding an isolate positive for multi-drug resistance, extended spectrum beta-lactamase or AmpC-producer phenotypes. Y-axis represents the percentage of all samples collected from that area that showed the resistance phenotype (±95% confidence intervals)

positive for *qnr* genes on PCR. Two were positive for *qnrS* and one was positive for *qnrB*. Among environmental samples isolates the *qnrB* was detected in *E. coli* from four (1·5%) samples.

Interestingly, the inhibitor-resistant and ESBL variant *bla*-TEM-158 was detected in *E. coli* isolated from 10 faecal samples and 1 environmental sample, all from the same practice.

Sequencing results are shown in Table 5. However, not all isolates positive for beta-lactamase genes returned sequences good enough for analysis, and these are recorded at the gene or group level only (e.g. CTX-M group 1)

DISCUSSION

Overall, in isolates from both faecal and environmental samples there were relatively high levels of resistance to a number of important antimicrobials including potentiated amoxicillin, fluoroquinolones and third generation cephalosporins. This has important implications for clinical veterinary practice and public health. Steps towards mitigating this problem will first require a thorough understanding of the prevalence and transmission in patients and the practice environment.

Table 5. Number and percentage of faecal (n=333) and environmental (n=257) samples with at least one *Escherichia coli* isolate having a resistance gene identified by sequence analysis

Gene	Faecal samples		Environmental samples	
	N	% (95% CI)	N	% (95% CI)
*bla*CTX-M-15 *†	20	6 (3·5 to 8·6)	8	3·1 (1 to 5·2)
*bla*CTX-M-1	1	0·3 (0 to 0·9)	1	0·4 (0 to 1·2)
*bla*CTX-M-9	1	0·3 (0 to 0·9)	0	
*bla*CTX-M-82	1	0·3 (0 to 0·9)	0	
*bla*TEM-158	10	3 (1·2 to 4·8)	1	0·4 (0 to 1·2)
*bla*SHV-12	0		2	0·8 (0 to 1·9)
*bla*CMY-2 †	72	21·6 (17·2 to 26·0)	22	8·6 (5·1 to 12·0)

*In five isolates positive on the universal *bla*CTX-M PCR the group was not identified
†Groups 1 and 9 *bla*CTX-M were detected in isolates by PCR but sequence analysis could not confirm the individual gene they carried belonging to these groups in five samples
‡All *citm* positive isolates that returned a result on sequence analysis corresponded to *bla*CMY-2

There was frequent potentiated-amoxicillin resistance among samples from all the practices, with an adjusted prevalence of 14% (albeit with some variation between practices). Potentiated amoxicillin is commonly used in companion animal practice in the UK (Mateus *et al.* 2011, Radford *et al.* 2011) and the frequent carriage of resistance is likely related to the frequency of use. Nevertheless, this is of some concern. In human medicine the prevalence of ESBLs in hospitals has been linked to use of potentiated amoxicillin in the community, highlighting the importance of interaction between community antimicrobial use and the development of AMR in hospitals (Aldeyab *et al.* 2012). Resistance to potentiated amoxicillin has been found in dogs in other studies, but at lower levels ranging from 6·3 to 8·3%. The high levels in this study are likely to reflect hospitalisation, because a study of non-vet visiting dogs in a similar area showed a relatively low level of potentiated amoxicillin resistance (Wedley *et al.* 2011).

The majority of *E. coli* with potentiated-amoxicillin resistance tested positive for *bla*AmpC genes, making AmpC production the most likely mechanism responsible for resistance. AmpC genes conferring AmpC production have also been found in clinical *E. coli* isolates from animals in the USA (Shaheen *et al.* 2011) and the Netherlands (Dierikx *et al.* 2012), and in hospitalised animals in Korea (So *et al.* 2012) and Australia (Sidjabat *et al.* 2006). High levels of AmpC-production have been found in a human healthcare setting where they were putatively linked with a high use of potentiated-amoxicillin in the same facility (Seiffert *et al.* 2013). These results suggest that AmpC-production is potentially an important mechanism conferring potentiated-amoxicillin resistance among hospitalised companion animals.

Potentiated-amoxicillin resistance and AmpC genes were found in environmental samples from all practices, although there was variation between practices, which followed a similar pattern to that seen in the faecal samples, suggesting a link between commensal faecal and environmental-resistant *E. coli*. Colonisation of dogs and humans and contamination of the veterinary environment by the same AmpC-producing *E. coli* strains has been reported previously (Sidjabat *et al.* 2006) and the environmental prevalence (4·1%) reported is similar to levels found in most of the practices in this study.

The prevalence of resistance to ciprofloxacin (a marker for fluoroquinolone resistance) was variable between practices, ranging from 8 to 44%. In contrast, ciprofloxacin resistance was only found in 2·2% of community dogs using similar methods in the same area (Wedley *et al.* 2011). Moreover, 73% of the 80 faecal samples with ciprofloxacin-resistant *E. coli* demonstrated MDR phenotypes. Fluoroquinolones are a class of antimicrobial used in treatment of important infections in both human and veterinary medicine and are classed as a high priority antimicrobial (WHO 2011), and resistance to these drugs is a serious concern for animal and public health. The frequent co-association of fluoroquinolone resistance with resistance to other, unrelated classes of antimicrobial is an additional concern. The *qnr* genes were detected at low rates among both faecal and environmental samples and are therefore unlikely to be a significant contributor to the levels of quinolone resistance observed in this study. Further mechanisms for quinolone resistance in this study were not studied, but they are most likely to be due to chromosomal mutations in the gyrase genes.

The prevalence of MDR in faecal samples from this study was 13%. The similar resistance profiles between MDR faecal and environmental samples are suggestive of cross contamination between animals and their environment. Of particular concern is the relatively frequent isolation of bacteria resistant to all seven antimicrobials tested. Other studies reported MDR in 48% in hospitalised dogs and 32% of stray dogs from Korea (Nam *et al.* 2010), 15% of community-based dogs in the UK (Wedley *et al.* 2011) and 9% of animals at admission in the USA (Hamilton *et al.* 2013). It has also been shown that resistant organisms are more prevalent among hospitalised or sick animals compared to non-hospitalised or healthy animals (Nam *et al.* 2010, Sun *et al.* 2010, Gibson *et al.* 2011). It is also noteworthy that the MDR isolates in this study frequently included resistance to potentiated amoxicillin and fluoroquinolones, whereas this was uncommon in MDR isolates from community-based dogs (Wedley *et al.* (2011), which were most frequently resistant to ampicillin, tetracycline and trimethoprim.

Resistance to third generation cephalosporins was detected at relatively high rates in this study. Studies of companion animals have found variable levels of resistance depending on setting and methods used: ranging from 60·5% in hospitalised pets treated with antimicrobials in China (Lei *et al.* 2010); 13% in dogs and cats from the community and nursing homes in Switzerland (Gandolfi-Decristophoris *et al.* 2013); and 2·4% from stray and hospitalised dogs in Korea (Nam *et al.* 2010).

The confirmed sample prevalence of ESBL-producer phenotypes in this study was 13%. ESBL production has been detected in: 54·5 and 24·5% in sick and healthy animals, respectively, in veterinary hospitals in China (Sun *et al.* 2010); 33·3% in Korean veterinary hospitals (So *et al.* 2012); 5% of faecal samples from cats and dogs in shelters in Germany (Franiek *et al.* 2012); and 16% of faecal samples from healthy cats and dogs in Tunisia (Ben Sallem *et al.* 2013). ESBLs have also been detected from clinical urinary isolates in the USA (O'Keefe *et al.* 2010, Shaheen *et al.* 2011) and Switzerland (Huber *et al.* 2013). There is clearly variation in ESBL-producer prevalence by location and setting. Hos-

pitalised animals appear to be at greater risk, though this study demonstrated variation among hospitals and there are likely to be hospital level factors that have an important influence. Assessing ESBL production by phenotypic results only led to an underestimation of the true prevalence in this study due to masking by AmpC-producer phenotypes in the same isolates. This was particularly of note in the practice that had high levels of AmpC production, demonstrating the importance of production of AmpC not only in the resistance it confers but also in its ability to make detecting and confirming ESBL production more difficult.

The most common ESBL genotype identified in this study was $bla_{CTX-M-15}$. Several other studies have found CTX-M-1 production to be more common in companion animals isolates (Costa *et al.* 2008, Dierikx *et al.* 2012). However, others have identified $bla_{CTX-M-15}$ in companion animals (Sun *et al.* 2010, Dierikx *et al.* 2012, So *et al.* 2012, Huber *et al.* 2013, Belas *et al.* 2014) and in Europe it is the second most commonly isolated ESBL gene from companion animals (Ewers *et al.* 2012). A number of ESBL-positive samples in this study were found to carry *bla*CTX-M genes, but these could not be assigned to a group (n=5 *E. coli*) or, when assigned to a group, the sequence was poor so that it could not be used to determine the specific bla_{CTX-M} gene type. The true prevalence of CTX-M type ESBLs may therefore be different. The $bla_{CTX-M-15}$ gene is the most common gene associated with the human pandemic O25/ST131 *E. coli* clone in the UK (Lau *et al.* 2008); however, no *E. coli* isolates that carried bla_{CTX-M} genes in this study were positive for markers for this clone. However, other *E. coli* clones have been isolated from companion animals in Europe associated with CTX-M production, such as ST648 (Ewers *et al.* 2014).

The $bla_{TEM-158}$ gene was the second most prevalent ESBL genotype found in this study. It was first detected from a faecal sample from a human intensive care patient in France and demonstrates both ESBL and inhibitor resistant TEM (IRT) characteristics (Robin *et al.* 2007), and due to this is often referred to as a complex mutant TEM (CMT). Other studies have identified TEM-158 production in urinary *E. coli* isolates in the human community in Morocco (Barguigua *et al.* 2013) and clinical isolates from human patients in Kenya (Kiiru *et al.* 2012). Evolution of TEM-158 has been shown to occur as a result of antimicrobial therapy in humans (Jacquier *et al.* 2013). Occurrence of this beta-lactamase at relatively high levels in one hospital is of concern given that it confers a wider spectrum of resistance to beta-lactams and the fact that its inhibitor resistant characteristics can make identification difficult. Molecular methods including sequencing are required to distinguish TEM-158 production from that of AmpC and other ESBL variants. We are not aware of any other studies in companion animals that have identified the presence of $bla_{TEM-158}$. As the only studies reporting this previously were from humans it may be that inter-species transmission has occurred or, alternatively, it may have been driven by antimicrobial therapy. Further study is needed to determine factors responsible for this, including examining the referring population of animals in the community.

Contamination of the human hospital environment with ESBL-producing *Enterobacteriaceae* originating from patients

has been reported (Guet-Revillet *et al.* 2012). Furthermore, in a companion animal veterinary hospital in Canada environmental contamination with *E. coli* was detected in 92% and CMY-2 (AmpC) producing *E. coli* in 9% of the hospitals sampled (Murphy *et al.* 2010). These findings suggest that *E. coli* with important resistance phenotypes such as EBSL and AmpC producers, are present in practice environments, where they may act as a source of infection and reservoir of resistance determinants.

For most practices the outside walking and toileting area were the areas with the most frequent isolation of resistant bacteria, despite practice staff picking up and disposing of faeces. Contamination of this area with *E. coli* may be associated with large numbers of dogs, mixing from different wards and difficulty with disinfection of the surface. Practices 1, 2 and 5 had grassy walking areas and practice 4 had a wood chip surface, which all would be difficult to disinfect. Only practice 3 had a concrete surface that should be easier to disinfect, and the isolation rate from the outside area in this practice was similar to other areas within the practice. Internal floors were next most frequently associated with resistant bacteria, with tables and keyboards less commonly contaminated. This may be associated with effective hand hygiene and/or the relative ease of cleaning and disinfecting these sites.

One weakness of this study was the variable number of samples returned by practices because of low throughput of animals and a lack of hospitalised patients in some hospitals. In addition, for ethical reasons, only naturally-voided faecal samples were collected, and animals that did not defecate (e.g. when hospitalised for short periods or if non-ambulatory) may be underrepresented. There was also some variation in time between sample collection and processing, although this never exceeded 3 days. The use of bags to collect faeces may have led to underrepresentation of soft or liquid faeces, therefore diarrhoeic dogs may also be underrepresented. Cats were included as there have been no equivalent studies of their carriage of resistant bacteria, but the study power was limited by low numbers associated with less frequent hospitalisation of cats. Environmental sample collection was variable as this was designed to fit around practice routine to minimise disruption. It is therefore possible that cleaning may have occurred at variable times before sample collection, which could have affected isolation rates of *E. coli*. Furthermore, only potential ESBL/AmpC producers were isolated following enrichment of environmental samples. Therefore, cephalosporin isolation rates and phenotypes are only directly comparable between faecal and environmental samples. Our study also used methodology which specifically selected for resistant variants of *E. coli*, so our reported prevalence may be higher compared to other studies in which selective isolation was not employed. Nevertheless because of this our findings are likely to reflect the true prevalence concerning faecal carriage of resistant bacteria in hospitalised animals. Finally, this study was based in secondary and tertiary referral hospitals, and the results may not reflect first opinion practice.

In summary, this project demonstrated that important AMR, MDR and ESBL phenotypes and genotypes are present among faecal and environmental *E. coli* in veterinary hospitals, including the detection of production of the IRT TEM-158. Such *E.*

coli are potential pathogens (Steen & Webb 2007, O'Keefe *et al.* 2010, Timofte *et al.* 2011, Huber *et al.* 2013, Nebbia *et al.* 2014), and treatment options for infections would be limited. Moreover, close contact between pets and their owners (Westgarth *et al.* 2008) could result in zoonotic transmission of resistant organisms or resistance determinants if present on mobile genetic elements. Contamination of the practice environment with these bacteria is of concern because environmental bacteria could be disseminated to new sites and animals (particularly vulnerable patients). They may also act as a source of infection for new animals in the environment and a source of resistance genes for exchange with previously susceptible *E. coli*. Further work is required to study the transmission of such resistant bacteria in companion animals and their practice environment, the resistance determinants and their mobility, and their association with virulent clones, to show how this contributes to clinical infections with resistant organisms. Furthermore determining risk factors associated with environmental contamination and carriage of resistant commensal *E. coli* and elucidating reasons for the differences between practices will help inform effective infection control measures and antimicrobial stewardship programmes.

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Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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