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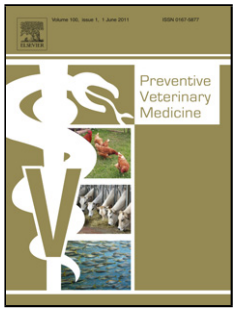

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Antimicrobial resistance risk factors and characterisation of faecal *E. coli* isolated from healthy Labrador retrievers in the United Kingdom

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Abstract

Antimicrobial resistant bacteria are increasingly detected from canine samples but few studies have examined commensal isolates in healthy community dogs. We aimed to characterise faecal *Escherichia coli* from 73 healthy non-veterinarian-visiting and non-antimicrobial treated Labrador retrievers, recruited from dog shows in the North West United Kingdom between November 2010 and June 2011. Each enrolled dog provided one faecal sample for our study. *E. coli* were isolated from 72/73 (99%) faecal samples. Disc diffusion susceptibility tests were determined for a range of antimicrobials, including phenotypic extended-spectrum beta-lactamase (ESBL) and AmpC-production. PCR assay detected phylogenetic groups and resistance genes ($bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, bla_{OXA}, bla_{CIT}, *qnr*), and conjugation experiments were performed to investigate potential transfer of mobile genetic elements. Multivariable logistic regression examined potential risk factors from owner-questionnaires for the presence of antimicrobial resistant faecal *E. coli*. Antimicrobial resistant, multi-drug resistant (≥ 3 antimicrobial classes; MDR) and AmpC-producing *E. coli* were detected in 63%, 30% and 16% of samples, respectively. ESBL-producing *E. coli* were detected from only one sample and conjugation experiments found that $bla_{\text{CTX-M}}$ and bla_{CIT} were transferred from commensal *E. coli* to a recipient strain. Most isolates were phylogenetic groups B1 and A. Group B2 isolates were associated with lower prevalence of resistance to at least one antimicrobial ($P < 0.001$) and MDR ($P < 0.001$). Significant at $P \leq 0.003$, was the consumption of raw meat for clavulanate-amoxicillin (OR: 9.57; 95% CI: 2.0-45.7) and third generation cephalosporin resistance (3GCR) (OR: 10.9; 95% CI: 2.2-54.0). AMR *E. coli* were surprisingly prevalent in this group of non-antimicrobial treated and non-veterinarian-visiting dogs and consumption of raw meat was a significant risk factor for antimicrobial resistance. These findings are of concern due to the increasing popularity of raw-meat canine diets, and the potential for opportunistic infection, zoonotic transmission and transmission of antimicrobial resistant determinants from commensal isolates to potential pathogenic bacteria.

Keywords: E. coli, Dogs, Multi-drug resistance, ESBL, AmpC, Phylogenetic group

1. Introduction

Escherichia coli is the main aerobe of the gastro-intestinal flora in humans and other animals (Tenaillon et al., 2010), and has been widely studied as an indicator of antimicrobial selection pressure (Gronvold et al., 2010). A stable gastrointestinal flora is important for health and acts as a colonisation barrier against pathogens (Vollaard and Clasener, 1994; Dethlefsen et al., 2007); this may be disturbed by a number of factors including disease, diet and antimicrobial therapy (Stecher and Hardt, 2008; Jernberg et al., 2010; Vincent et al., 2010).

Of particular concern is the emergence and dissemination of extended spectrum beta-lactamase (ESBL) and AmpC-producing *E. coli* that are resistant to a variety of beta-lactam antimicrobials including third generation cephalosporins (3GCR) (Livermore and Hawkey, 2005; Thomson, 2010). Genes encoding these enzymes are carried on plasmids, often in conjunction with other antimicrobial resistance determinants, enabling horizontal transmission of multidrug resistance (MDR) (Zhao et al., 2001; Li et al., 2007; Karczmarczyk et al., 2011; Dahmen et al., 2012). ESBL-, AmpC-producing and MDR *E. coli* have been detected in healthy (De Graef et al., 2004; Carattoli et al., 2005; Costa et al., 2008; Wedley et al., 2011) and sick dogs (Carattoli et al., 2005; Pomba et al., 2009; Gibson et al., 2011a), and increased detection has been associated with exposure to antimicrobials and veterinary healthcare (Moreno et al., 2008; Damborg et al., 2011; Gibson et al., 2011a, b).

E. coli populations include commensal and pathogenic strains. Compared to commensal strains, pathogenic strains are more likely to carry a range of virulence genes that can facilitate disease (Johnson and Russo, 2002; Nowrouzian et al., 2006). Gut colonisation by ExPEC (extra-intestinal pathogenic *E. coli*) strains is a prerequisite for extra-intestinal infections. The gut of healthy

humans and other animals can be a reservoir of ExPEC strains (Johnson et al., 2003; Russo and Johnson, 2003), which are potentially zoonotic (Johnson et al., 2009), and may be shared between humans and pets within households (Johnson et al., 2008). Food, particularly chicken meat, is also a potential source of ExPEC strains for humans and dogs (Johnson et al., 2007; Johnson et al., 2009; Vincent et al., 2010).

Phylogenetic grouping is a simple and inexpensive method to investigate the genetic background, potential pathogenicity, and antimicrobial resistance traits of *E. coli* isolates (Sato et al., 2014). A PCR assay to assign *E. coli* isolates to four major phylogenetic groups: A, B1, B2 and D (Clermont et al., 2000) has been widely used and recently updated (Doumith et al., 2012). Additionally, the original method (Clermont et al., 2000) has been revised (Clermont et al., 2013) to assign isolates to eight different phylogenetic groups: A, B1, B2, C, D, E, F and *Escherichia* Clade I. Phylo-groups B2, D, E and F are more likely to be involved in extra-intestinal infections compared to A, B1 or C (Picard et al., 1999; Moissenet et al., 2010; Tenaillon et al., 2010) and Clade isolates are thought to reside outside of the gut (Walk et al., 2009).

The distribution of these phylo-groups amongst different hosts is not heterogeneous and may depend on characteristics such as body mass, diet and environment (Gordon and Cowling, 2003; Escobar-Paramo et al., 2004; Tenaillon et al., 2010). In healthy humans, phylo-group A generally predominates followed by B2, B1 and D, whereas in animals group B1 predominates followed by A, B2 and D (Tenaillon et al., 2010). Diversity due to host diet has also been reported with group A predominating in carnivores and omnivores, and group B1 in herbivores (Escobar-Paramo et al., 2006; Baldy-Chudzik et al., 2008; Carlos et al., 2010).
Antimicrobial resistance has been linked to the non-B2 phylo-groups in people, cattle, pigs and dogs (Johnson et al., 2003; Moreno et al., 2008; Johnson et al., 2009). In dogs, phylo-group D isolates are more likely to be antimicrobial resistant, including fluoroquinolone, 3GCR and MDR (Platell et al., 2011; Tamang et al., 2012; Sato et al., 2014) and group B2 are more likely to be

antimicrobial susceptible (Johnson et al., 2009; Platell et al., 2011; Sato et al., 2014). However ESBL-producing fluoroquinolone resistant and MDR ExPEC strains, that further challenge therapeutic regimes are emerging amongst human clinical isolates, and have been reported in dogs (Russo and Johnson, 2003; Johnson et al., 2009; Platell et al., 2010).

Previous studies have concentrated on clinical isolates and the effects of potential risk factors, in particular antimicrobial pressure. However, few studies have examined canine gastrointestinal *E. coli* populations under natural conditions. The aim of this study was to determine the prevalence of antimicrobial resistance and determine phylogenetic groups amongst faecal *E. coli* from a group of healthy non-vet visiting and non-antimicrobial treated dogs. In addition, we aimed to examine the association of these findings with potential risk factors.

2. Methods

2.1 Study Population

The prevalence of antimicrobial resistant faecal *E. coli* in healthy non-antimicrobial treated and non-veterinarian-visiting dogs was hypothesised to be low. Simple sample size estimates to determine prevalence showed that with an expected prevalence of 5%, precision of 5% and 95% confidence, 73 dogs would be required. Labrador retriever dogs were recruited from two dog shows in the North West UK between November 2010 and June 2011. One healthy dog of any age was enrolled from each household following a clinical examination. Dogs that had received topical or systemic antimicrobial therapy, had been admitted to a veterinary clinic within the last 12 months, or were determined not to be healthy were excluded. All dog owners gave written informed consent before enrolment in this study and completed a two-page questionnaire regarding potential risk factors for the carriage of antimicrobial resistant bacteria that was administered at recruitment by a veterinarian. Time to complete the questionnaire was

1-2 minutes and it was either submitted at recruitment or returned with the sample by first-class post. The questionnaire had been previously used (Wedley et al., 2014) and consisted of simple closed questions with tick box responses and space for additional information. A "Don't Know" response was included for all questions to enable the respondent to avoid answering incorrectly if they were uncertain.

Data were collected regarding patient signalment and diet, the presence, number and type of in-contact pets, previous medical history of the household (including antimicrobial therapy or hospitalisation of people or other pets), and whether household members worked with farm animals or in healthcare. The University of Liverpool, School of Veterinary Science Ethics-Committee approved the study protocol.

2.2 Specimen collection and bacterial isolation

The next fresh faecal sample was collected in a sterile pot and submitted at enrolment or returned by first-class post. Faecal samples were mixed with an equal volume of brain heart infusion broth with 5% glycerol (BHI-G) on receipt. Each faecal homogenate was streaked onto one eosin methylene blue agar (EMBA) plate without antimicrobials, one EMBA plate impregnated with 1 μg/ml ceftazidime (CZ) and one EMBA plate impregnated with 1 μg/ml cefotaxime (CX) (Liebana et al., 2006). In addition, one EMBA plate and one MacConkey's agar (MAC) plate were inoculated with the faecal homogenate for confluent bacterial growth and seven antimicrobial discs [(10 μg ampicillin (Amp), 30 μg clavulanate-amoxicillin (AC), 1 μg ciprofloxacin (Cip), 30 μg chloramphenicol (Chl), 30 μg nalidixic acid (Nal), 30 μg tetracycline (Tet) and 2.5 μg trimethoprim (Tm)] were applied (Bartoloni et al., 2006). A further 500 μL of faecal homogenate was enriched in 4.5 ml of buffered peptone water and plated onto EMBA plates impregnated with CZ and CX, as above. All plates and broths were incubated aerobically for 18 - 20 hours at 37°C. Three colonies, whose morphology resembled *E. coli,* were selected from plain EMBA. One colony growing within the zone of inhibition around each antimicrobial disc on both the EMBA and MAC and/or from the CX and/or CZ plates was selected if present.

Colonies were sub-cultured onto nutrient agar and incubated aerobically for 18 - 20 hours at 37°C. Gram stains and biochemical tests (catalase production, lack of oxidase, lactose fermentation, indole production and inability to use citrate as a carbon source) to confirm *E. coli* were performed on fresh overnight cultures. All antimicrobial discs were obtained from MAST Group Ltd., Liverpool, UK, and the media from LabM Ltd, Bury, UK, and the CX and CZ powder from Sigma-Aldrich Company Ltd., Gillingham, UK.

2.3 Antibiotic susceptibility testing

Antimicrobial susceptibility disc diffusion testing was performed according to British Society for Antimicrobial Chemotherapy guidelines (BSAC; Version 11.1 May 2012) (Andrews and Testing, 2007) and the same panel of seven antimicrobial discs were applied. Plates were incubated aerobically at 37°C for 18 - 20 hours. *E. coli* ATCC® 25922 (LGC Standards, Teddington, UK) cultured overnight on nutrient agar at 37°C was used as a control.

2.4 Screening for phenoptypic AmpC- and ESBL-producing E. coli

Isolates with beta-lactam resistance were tested for ESBL production (Extended Spectrum Beta-Lactamase Set, MAST Group Ltd., Liverpool, UK) (M'Zali et al., 2000) and AmpC production (AmpC detection set, MAST Group Ltd., Liverpool, UK) (Halstead et al., 2012). The plates were incubated aerobically at 37°C for 18 - 20 hours. ESBL production was confirmed when the zone around the cephalosporin disc was expanded in the presence of the clavulanic acid by a minimum of 5 mm for ≥ 1 antimicrobial pairs. AmpC production was confirmed when the zone of inhibition around disc C was greater than that for discs A and B by a minimum of 5 mm. *E. coli* ATCC® 25922 (LGC Standards, Teddington, UK) cultured overnight on CAB at 37°C was used as a control. All confirmed *E. coli* isolates were stored at – 80°C in Microbank vials (Pro-Lab, Bromborough, UK) for further analysis.

2.5 Genotypic identification of E. coli and characterisation of resistance genes

PCR assays for the *uid*A gene (McDaniels et al., 1996) were used to confirm *E. coli*. Isolates with phenotypic ESBL or AmpC-production were tested for the presence of $bla_{\text{CTX-M}}$ (Batchelor et al., 2005), bla_{SHV}, bla_{TEM} *and* bla_{OXA} (Dallenne et al., 2010). Isolates with $bla_{\text{CTX-M}}$ were tested for CTX-M group 1, 2 and 9 genes (Batchelor et al., 2005; Hopkins et al., 2006). Phenotypic AmpC producers were tested for bla_{AmpC} gene (Perez-Perez and Hanson, 2002). All isolates were tested for the presence of *qnr*A, *qnr*B or *qnr* S genes (Robicsek et al., 2006). PCR assays were performed with 5 µL of bacterial DNA, 5 pmol of each primer, 4 µL of 5x FIREPol® Master Mix (12.5 mM MgCl2), 0.5 µl of FIREPol® DNA Polymerase 5 U/µl (Solis-Biodyne, Tartu, Estonia) and water to made up to a total reaction volume of 25 µL. PCR products were analysed by agarose gel (1.5%) electrophoresis and the DNA fragments were visualised under UV light after peqGREEN (Peqlab, Fareham, UK) staining.

2.6 Phylogenetic groups

E. coli isolates were segregated into phylogenetic groups by PCR assay. In short, a multiplex PCR for phylo-groups A, B1, B2 and D (Doumith et al., 2012) and for phylo-groups A, B1, B2, D, C, E, F and Clade I (Clermont et al., 2013) were performed.

2.7 Conjugation experiments

MDR isolates, including two ESBL- and three AmpC-producing isolates, were tested for the ability to transfer antimicrobial resistance determinants as previously described (Karczmarczyk et al., 2011). Briefly, overnight cultures of the donor and recipient strains grown in 5 ml of nutrient broth were mixed and incubated at 37°C for 18 hours. The transconjugants were selected on MacConkey agar supplemented with 100 µg/ml rifampin along with 50 µg/ml ampicillin, 50 µg/ml nalidixic acid, 30 µg/ml tetracycline, 50 µg/ml trimethoprim or 1 µg/ml of cefotaxime (Sigma-Aldrich, UK). If present, up to three lactose-negative colonies were selected from each plate onto nutrient agar and incubated at 37°C for 18 hours. Transconjugates underwent antimicrobial susceptibility tests and PCR assay for bla_{AmpC} and $bla_{\text{CTX-M}}$.

206

207 *2.8 Statistical analysis*

208 Outcome data for antimicrobial resistance phenotype were collapsed to the sample level such
209 that a sample with at least one isolate that was resistant was classed as resistant for analysis.
210 Independent variables were created from the owner questionnaires. Except for the age of the
211 dog, all variables were dichotomous (Supplementary table 1). The six antimicrobial resistant
212 outcomes considered were: AMR (any resistance), clavulanate-amoxicillin (ACR), ciprofloxacin
213 (CipR), third generation cephalosporin (3GCR), beta-lactam resistance (BLR) and MDR
214 (resistance to ≥ 3 antimicrobial classes). Each resistance outcome was a yes/no variable.
215 Logistic regression examined the association between independent variables and all outcomes.
216 All variables were analysed in a univariable models and tested in multivariable models if *P*-
217 value < 0.25. Final models were constructed by manual backwards stepwise procedures where
218 variables with a likelihood ratio *P*-value < 0.05 were retained. Prior to inclusion in the models,
219 age was categorised and each category was confirmed for linearity of the odds ratio. Collinearity
220 between explanatory variables was assessed using two-by-two tables and Pearson's Chi-square
221 test for independence (if N > 5), otherwise Fisher's exact tests were used. For variables with a
222 significant association ($P < 0.05$) only variables with the lowest *P*-value were considered for
223 inclusion in the multivariable models. *Sidak-Bonferroni* (Keppel, 2004) correction was used to
224 calculate a corrected *P*-value based on testing six antimicrobial resistance outcomes; hence
225 significance was set at $P < 0.0085$.

226

227 To examine the agreement of the published between two published methods for determining *E.*
228 *coli* phylogenetic groups at the isolate level (Doumith et al., 2012; Clermont et al., 2013), a *kappa*
229 statistic was calculated (Landis and Koch, 1977). Confidence intervals were calculated and
230 Fisher's exact tests were used to examine the association between antimicrobial resistance
231 outcome and phylogenetic group at the isolate level detected by the Doumith et al. (2012)
232 method. The corrected *P*-value (calculated as above) for 24 tests was 0.002.

Tests for *kappa* statistic, Pearson's Chi-square and binary logistic regression were performed using SPSS software package (SPSS 20.0 for Mac, SPSS Inc, Chicago, Illinois). Tests for Fisher's exact were performed using GraphPad Software 2014 (http://graphpad.com/quickcalcs/contingency1.cfm).

Results

3.1 Study population

In total, 73 Labrador retriever dogs were recruited providing one sample each: 3 to 12-months-old (n = 21 dogs), >12 months to 24-months-old (n = 25), and > 2-years-old (n = 25). There were 35 female and 38 male dogs. Dogs were from 23 counties of England, Northern Ireland, Scotland and The Isle of Man. The largest proportion (40%) was from the northwest of England.

3.2 Antimicrobial resistance

Faecal *E. coli* were isolated from 72 samples (99%; 95% CI: 92.6-99.8). Of these 46 samples had at least one AMR *E. coli* and 22 samples had at least one MDR isolate (Figure 1). A high prevalence of resistance to ampicillin and/or tetracycline and/or trimethoprim, and MDR was observed. Only one sample carried MDR (AmpR, 3GCR, TetR, TMR) ESBL-producing faecal *E. coli* (CTX-M group 1 and bla_{TEM}). Phenotypic AmpC-producing *E. coli* (n = 16 isolates) were detected in 12 samples (16%; 95% CI: 9.7-26.6), but only three samples carried bla_{AmpC} gene (all CIT). *E. coli* with bla_{SHV}, bla_{OXA} or *qnr* genes were not detected in any sample.

3.3 Phylogenetic groups

Table 1 shows the assignment of isolates to groups B1, B2, A and D for both methods (Doumith et al., 2012; Clermont et al., 2013). Most isolates were of B1, followed by A, B2 and D

phylogenetic groups. Approximately three quarters of the isolates originally assigned to group A by the Doumith method were reassigned to either group C (n = 39) or *Escherichia* Clades III-IV (n = 4 isolates) by the Clermont method; nine isolates that were initially assigned to either group B1 (n = 1), B2 (n = 3) or D (n = 5) were reassigned to group E. Three isolates, assigned to group D by the Doumith method, were reassigned to group F, and two isolates originally grouped as B1 were classed as unknown by the Clermont method. The agreement between the two methods was very good for the assignment of phylo-groups B1 and B2, but only fair or moderate for phylo-groups A and D, respectively (Landis and Koch, 1977).

3.1 Conjugation studies

In total, 23 isolates transferred resistance to the recipient strain including ESBL- and AmpC-producing *E. coli* (Table 2).

3.2 Association between isolate phylo-group and antimicrobial resistance

Table 3 shows that the majority of the isolates in phylo-groups A, B1 and D were resistant to at least one antimicrobial (AMR), particularly a beta-lactam. Group B2 did not contain isolates with clavulanate-amoxicillin resistance, 3GCR or MDR and group B2 was associated with significantly lower levels of AMR and MDR ($P < 0.001$) compared to the other groups. Ciprofloxacin resistance was spread between the different phylo-groups.

3.3 Logistic regression: antimicrobial resistance with questionnaire data

All of the dogs in the study were fed dry dog food; however some were supplemented with tinned dog food, home-cooked meats, proprietary dog treats, table scraps or raw meat. In addition, 42 dogs regularly scavenged from the environment. Clavulanate-amoxicillin resistance ($P = 0.003$) and 3GCR ($P = 0.002$) were associated with dogs eating raw meat (chicken, red-meat

and/or tripe), whilst consumption of proprietary dog treats appeared to be protective against ciprofloxacin (P = 0.003) and MDR (P = 0.001) *E. coli* (Table 4).

3. Discussion

This study found an unexpected high prevalence of AMR (including MDR and phenotypic AmpC-producers), amongst canine faecal *E. coli* from a group of healthy Labrador retrievers in the UK, in the absence of direct antimicrobial selective pressure and veterinary premises contact.

The prevalence of MDR in this study was 30% of dogs (95% CI: 19.6 – 40.6). A previous healthy dog study In North West UK reported a MDR prevalence of 15.3% of dogs (95% CI: 10.4-20.5) (Wedley et al., 2011). This variation may be due to the five-year difference in sample collection between the studies; prevalence of AMR may have increased in such populations over time, or it may be due to differences in the sampled populations. The dogs in this study were regularly in close contact, in confined spaces, with many other dogs and humans at dog shows all over the UK. Sharing of faecal *E. coli* isolates may occur between dogs and between dogs and humans in regular close proximity, including MDR resistant strains (Skurnik et al., 2006; Johnson et al., 2008; Damborg et al., 2009; Stenske et al., 2009; Harada et al., 2011), and dogs housed together in groups may have a higher prevalence of AMR faecal *E. coli* than individual dogs (De Graef et al., 2004).

Only 5% of the dogs in this study had 3GCR faecal *E. coli* with either $bla_{\text{CTX-M}}$ (1 dog) or bla_{AmpC} (three of the 12 dogs with phenotypic AmpC production), in agreement a low prevalence (4%) of healthy dogs with faecal *E. coli* harbouring these genes has previously been reported in the UK (Wedley et al., 2011). Antimicrobial therapy with enrofloxacin or cefalexin has been reported to select for faecal *E. coli* carrying $bla_{\text{CTX-M}}$ or bla_{AmpC} (Moreno et al., 2008; Damborg et al., 2011) and the absence of antimicrobial therapy of dogs in the current study may explain the

low prevalence. Ciprofloxacin resistance was uncommon, but when present corresponded with MDR. This finding has been previously reported amongst canine clinical isolates, where it was proposed that this was due to 'last-line' clinical use of fluoroquinolones in dogs (Platell et al., 2011).

Group B1 was the most common phylo-group detected, followed by A, B2 and D, which concurs with Tenaillon et al. (2010) who reviewed a number publications examining faecal *E. coli* from various animal species. Damborg et al. (2009) also reported a predominance of phylo-group B1 in dogs (n = 13). However, in other studies a predominance of group B1 followed by group A has been reported in farmed and wild herbivorous animals (Escobar-Paramo et al., 2006; Carlos et al., 2010), whereas omnivorous and carnivorous animals had a predominance of A followed by B1 (Escobar-Paramo et al., 2006). The base diet of the dogs in this study was proprietary dry dog food, consisting of a combination of crude fibre and protein, equivalent to an omnivorous diet. Hence, we expected the phylogenic diversity to be similar to that reported for other domesticated omnivorous animals. High levels of crude dietary fibre found in some dog foods could impact the abundance of certain phylo-groups, for example decrease cell densities of B2 strains compared to B1 strains (O'Brien and Gordon, 2011), although this variable was not examined in the current study. Phylo-group B1 predominate in soil, sediment and water, the secondary habitat of *E. coli* (Savageau, 1983; Walk et al., 2007), representing a potential source for dogs that frequently drink, scavenge and/or orally explore their environments.

Previous studies have reported that antimicrobial resistant *E. coli* from humans and dogs are less likely to be of phylo-group B2 (Johnson et al., 2003; Johnson et al., 2009; Platell et al., 2010; Platell et al., 2011; Sato et al., 2014). Similarly in this study, group B2 isolates were less likely to be antimicrobial resistant and there were no MDR, 3GCR or clavulanate-amoxicillin resistant isolates in this group. The majority of the phylo-group D isolates were resistant to at least one tested antimicrobial however this was not found to be statistically significant. Other studies

have reported a relationship between group D and fluoroquinolone resistance, 3GCR and MDR (Deschamps et al., 2009; Platell et al., 2011; Tamang et al., 2012; Sato et al., 2014). These associations were not made in this study, although this may have been affected by the low prevalence of these resistance outcomes in phylo-group D, in particular fluoroquinolone resistance.

There was very good agreement between the two methods for assigning isolates to groups B1 and B2 but only fair to moderate for groups A and D respectively. This was due to reassignment of group A strains to group C, and group D strains to groups B1, E and F. Other studies have compared multiplex PCR and MLST for phylogenetic grouping and reported 80-90% accuracy for the multiplex method; mostly due to agreement for groups B1 and B2 rather than A and D (Gordon et al 2008; Doumith et al 2012). Further comparison of the new Clermont multiplex PCR method with MLST for phylo-grouping may find improved accuracy for assignment of groups A and D. The new Clermont method for assigning phylogenetic groups classed 39 isolates as group C. Compared to commensal *E. coli* from other species (human, bird, non-human mammals) (Clermont et al., 2013) this phylo-group appears to be prevalent in this group of dogs, but further studies are required to confirm this finding. Phylo-groups E and F are potential ExPEC strains (Tenaillon et al., 2010) and were re-assigned from the other potential ExPEC groups, D and B2.

Multivariable analysis found a relationship between consumption of raw meat diets (chicken, red meat and/or tripe) and resistance to clavulanate amoxicillin or 3GCR. In contrast, consumption of proprietary dog treats appeared to be protective against ciprofloxacin resistance and MDR. Food, particularly chicken meat, has been reported as a possible source of antimicrobial resistant bacteria, including ExPEC, for humans and dogs (Johnson et al., 2007; Lefebvre et al., 2008; Johnson et al., 2009; Vincent et al., 2010). Both ESBL- and AmpC-producing, and ciprofloxacin resistant *E. coli* have been isolated from chickens and pigs in Spain,

and the predominant phylo-groups reported from chickens, pigs and cattle in the US, Spain and South Korea are B1, A and D (Johnson et al., 2003; Johnson et al., 2007; Unno et al., 2009; Cortes et al., 2010). Recommendations to feed raw meat diets are of some concern, as raw meats may be a source of AMR and/or pathogenic organisms that are potential animal and a public health risks and this needs further investigation (Johnson et al., 2008).

It is likely that the canine faecal flora consists of a variety of organisms in addition to canine resident bacteria, derived from in contact humans and animals, diet or the environment. Newly introduced bacteria may act as a reservoir of antimicrobial resistant determinants for resident bacteria even if only transient in the gut and selective pressure through antimicrobial therapy may facilitate establishment as permanent residents of the gut flora.

Limitations of this study include the low number of observations for some outcomes, which reduced the power of the study. Selection and testing of more isolates may have increased the detection of antimicrobial resistance, in particular ESBL-producing *E. coli.* However, despite the small subgroups, we were able to identify strong associations between outcomes and risk factors. It is possible that some isolates were not detected due to postal delivery and delayed processing. However (Maule, 2000) reported *E. coli* survival in aerated manure of at least a month. Another limitation was that the study only included one dog breed and was in a limited demographic area. It is possible that samples from other breeds and other geographical populations would differ.

4. Conclusion

In conclusion, the overall prevalence of AMR and MDR amongst canine faecal *E. coli* was higher than expected for a group of healthy non-veterinarian-visiting and non- antimicrobial-treated dogs. The predominant faecal *E. coli* phylogenetic group in these dogs was group B1, and group

B2 isolates were less likely than the other groups to harbour antimicrobial resistance, in agreement with previous work. In particular, consumption of raw meat diets were associated with increased detection of 3GCR and clavulanate-amoxicillin resistant canine faecal *E. coli* in this group of dogs, representing a potential animal welfare and zoonotic risk.

Conflict of interest

Vanessa Schmidt, Neil McEwan and Tim Nuttall have received other unrelated funding from Zoetis (previously Pfizer Animal Health UK). The authors declare that there are no financial or non-financial competing interests.

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Table 1. Cross tabulation of the results of *E. coli* isolates, from 74 dogs recruited from UK dog shows between November 2010 and June 2011, classified as phylogenetic groups A, B1, B2 or D by both Doumith et al 2012 and Clermont et al 2013 Multiplex PCR methods.

Table 2. Conjugation experiments: resistance phenotype and genes detected in 25 donor *E. coli* isolates, from 73 dogs recruited from UK dog shows between November 2010 and June 2011, and transconjugates.

The recipient strain was rifampin-resistant, lactose-negative *E. coli* 26R793. AMP = ampicillin; AC = clavulanate-amoxicillin; CIP = ciprofloxacin; CHL = chloramphenicol; NAL = nalidixic acid; TET = tetracycline; TM = trimethoprim; [1]present in donor and transconjugate.

Table 3. Cross tabulation of the 186 *E. coli* isolates, from 74 dogs recruited from UK dog shows between November 2010 and June 2011, in each of four phylogenetic group and each of six antimicrobial resistance outcomes.

ACR = clavulanate-amoxicillin resistance, CipR = ciprofloxacin resistance; BLR = beta-lactam resistance; 3GCR = third generation cephalosporin resistance, AMR = any antimicrobial resistance, MDR = resistance to ≥ 3 antimicrobial classes; n = total number of isolates in each phylogenetic group with each antimicrobial resistance outcome; % = percentage of isolates in each phylogenetic group with each antimicrobial resistance outcome; CI = 95% confidence interval; *P* = *P*-value is from Fisher's exact test (two-tailed); Significant set at $P < 0.002$ (*Sidak-Bonferroni* correction).

Table 4. Final multivariable logistic regression models for clavulanate-amoxicillin resistance, ciprofloxacin resistance, 3GCR and MDR outcomes for faecal *E. coli* in 73 samples from 73 dogs recruited from dog shows in the UK between November 2010 and June 2011.

[1] Reference category is the absence of the risk factor; [2]Within 12 months of enrolment; 3GCR = 3rd generation cephalosporin resistance; MDR = antimicrobial resistance to three or more antimicrobial classes; [3]Hosmer and Lemeshow Goodness-of-fit; *P*-value; Covariate *P* values are from the * Likelihood-ratio test statistic. Significant set at $P < 0.0085$ (*Sidak-Bonferroni* correction).

Figure 1. The percentage of 73 samples with at least one faecal *E. coli* with resistance to the tested antimicrobials for 73 dogs (95% CI) recruited from UK dogs shows between November 2010 and June 2011.

AmpR = ampicillin resistance; ACR = clavulanate-amoxicillin resistance; CipR = ciprofloxacin resistance; NalR = nalidixic acid resistance; ChlR = chloramphenicol resistance; TetR = tetracycline resistance; TmR = trimethoprim resistance; 3GCR = third generation cephalosporin resistance; BLR = beta-lactam resistance; AMR = resistance to at least one tested antimicrobial; MDR = multidrug resistance (resistance to three or more antimicrobial classes)

Table 1. Cross tabulation of the results of *E. coli* isolates, from 73 dogs recruited from UK dog shows between November 2010 and June 2011, classified as phylogenetic groups A, B1, B2 or D by both Doumith et al 2012 and Clermont et al 2013 Multiplex PCR methods.

			Clermont A		
			No	Yes	Total
Doumith A	No	Number of isolates	128	2	**130**
	Yes	Number of isolates	43	14	**57**
Total		Number of isolates	**171**	**16**	**187**

Kappa = 0.29; SE: 0.07, P < 0.001

			Clermont B1		
			No	Yes	Total
Doumith B1	No	Number of isolates	105	4	**109**
	Yes	Number of isolates	5	73	**78**
Total		Number of isolates	**110**	**77**	**187**

Kappa = 0.9; SE: 0.03, P < 0.001

			Clermont B2		
			No	Yes	Total
Doumith B2	No	Number of isolates	154	2	**156**
	Yes	Number of isolates	3	28	**31**
		Total number of isolates	**157**	**31**	**187**

Kappa = 0.9; SE: 0.043, P < 0.001

			Clermont D		
			No	Yes	Total
Doumith D	No	Number of isolates	167	0	**167**
	Yes	Number of isolates	13	7	**20**
Total		Number of isolates	**180**	**7**	**187**

Kappa = 0.49; SE: 0.12, P < 0.001

Table 2. Conjugation experiments: resistance phenotype and genes detected in 25 donor *E. coli* isolates, from 73 dogs recruited from UK dog shows between November 2010 and June 2011, and transconjugates.

Donor resistance phenotype	Transconjugate resistance phenotype	bla_{CIT} or bla_{CTX-M} gene[1]
AMP, TET, TM	AMP, TET, TM	
AMP, CHL, TET, TM	AMP, TET, TM	
AMP, CHL, TET, TM	AMP, CHL, TET, TM	
AMP, TET, TM	TET	
AMP, CHL, TET, TM	AMP, TET	
AMP, TET, TM	AMP, TET, TM	
AMP, TET, TM	AMP, TET, TM	
AMP, CIP, TET, TM	AMP, TET, TM	
AMP, AC, CIP, CHL, NAL, TET, TM	AMP	bla_{CIT}
AMP, CHL, TET	AMP, CHL, TET	
AMP, CHL, TET	AMP, CHL, TET	
AMP, CHL, NAL, TET, TM	AMP, TET, TM	
AMP, CIP, CHL, NAL, TET, TM	AMP, CHL, TET, TM	
AMP, CHL, TET	AMP, CHL, TET	
AMP, TET, TM	AMP, TET, TM	
AMP, AUG, CHL, TM	AMP, TM	
AMP, CHL, TET, TM	AMP	
AMP, CIP, CHL, NAL, TET, TM	AMP, CHL, TET, TM	
AMP, CHL, TET	AMP, CHL, TET	
AMP, CHL, TET	AMP, CHL, TET	
AMP, CHL, TET	AMP, CHL, TET	
AMP, CHL, TET	AMP, CHL, TET	
AMP, CHL, TET	AMP, CHL, TET	
AMP, CIP, CHL, NAL, TET, TM	AMP, CHL, TET, TM	
AMP, TET, TM	AMP, TET, TM	bla_{CTX-M}

The recipient strain was rifampin-resistant, lactose-negative *E. coli* 26R793. AMP = ampicillin; AC = clavulanate-amoxicillin; CIP = ciprofloxacin; CHL = chloramphenicol; NAL = nalidixic acid; TET = tetracycline; TM = trimethoprim; [1]present in donor and transconjugate.

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Resistance Outcome	Phylogenetic group															
	A (n = 57)				B1 (n = 78)				B2 (n = 31)				D (n = 20)			
	n	%	CI	*P*	n	%	CI	*P*	n	%	CI	*P*	n	%	CI	*P*
ACR (n = 24)	6	11	2.6 - 18.5	0.6	11	14	6.4 – 21.8	0.7	0	0	0	0.016	7	35	14 – 55.9	0.006
CipR (n = 19)	8	14	5.0 – 23.0	0.3	8	10	3.5 – 17.0	0.9	2	6	0 - 15	0.75	1	5	0 – 14.6	0.7
BLR (n = 109)	35	61	48.8 – 74.0	0.6	48	62	50.7 – 72.3	0.5	12	39	21.6 – 55.9	0.027	15	75	56 – 94.0	0.15
3GCR (n = 19)	5	9	1.4 – 16.1	0.8	8	10	3.5 – 17.0	0.9	0	0	0	0.4	6	30	9.9 – 50.1	0.008
AMR (n = 148)	49	86	76.9 – 95.0	0.2	65	83	75.1 – 91.6	0.3	17	55	37.3 – 72.4	< 0.001	19	95	85.5 – 100	0.08
MDR (n = 61)	24	42	29.3 – 54.9	0.09	31	40	8.9 – 50.6	0.08	0	0	0	< 0.001	6	30	9.9 – 50.1	0.99

677 ACR = clavulanate-amoxicillin resistance, CipR = ciprofloxacin resistance; BLR = beta-
678 lactam resistance; 3GCR = third generation cephalosporin resistance, AMR = any
679 antimicrobial resistance, MDR = resistance to ≥ 3 antimicrobial classes; n = total number
680 of isolates in each phylogenetic group with each antimicrobial resistance outcome; % =
681 percentage of isolates in each phylogenetic group with each antimicrobial resistance
682 outcome; CI = 95% confidence interval; *P* = *P*-value is from Fisher's exact test (two-
683 tailed); Significant set at $P < 0.002$ (*Sidak-Bonferroni* correction).

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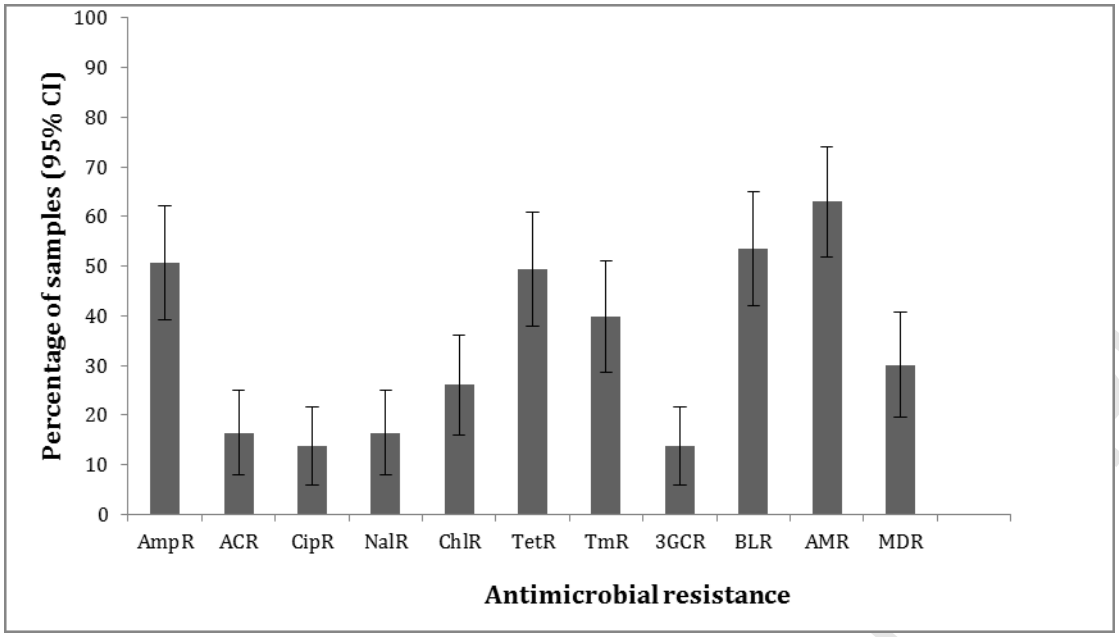
Table 4. Final multivariable logistic regression models for clavulanate-amoxicillin resistance, ciprofloxacin resistance, 3GCR and MDR outcomes for faecal *E. coli* in 73 samples from 73 dogs recruited from dog shows in the UK between November 2010 and June 2011.

Resistance outcome	Covariates	B	SE (b)	Adjusted OR	95% CI	*P*-value*
Clavulanate-amoxicillin						
P = 0.8[3]	Dog eats raw meat[1]	2.3	0.8	**9.57**	2.0 - 45.7	0.003
	In-contact had antimicrobials[2]	-1.6	0.8	0.19	0.04 - 0.9	0.028
	Constant	-1.5	0.5			0.005
Ciprofloxacin						
P = 0.99 [3]	Dog fed treats[1]	-1.9	0.8	**0.1**	0.03 - 0.6	0.007
	Dog eats raw meat[1]	0.9	0.8	**2.6**	0.5-12.2	0.25
	Constant	-0.6	0.5			0.19
3GCR						
P = 0.9 [3]	Dog eats raw meat[1]	2.4	0.8	**10.9**	2.2 – 54.0	0.002
	In contact had antimicrobials[1]	-1.6	0.8	0.2	0.04 - 0.9	0.05
	Multi-animal household[1]	1.6	0.9	5.1	1.0 - 26.5	0.04
	Constant	-2.4	0.7			0.001
MDR						
P = 0.5 [3]	Dogs fed treats[1]	-1.9	0.6	**0.16**	0.04 - 0.5	0.001
	Owner works with farm animals[1]	-1.5	0.9	0.2	0.04 - 1.3	0.07
	Constant	0.7	0.5			0.16

[1] Reference category is the absence of the risk factor; [2]Within 12 months of enrolment; 3GCR = 3rd generation cephalosporin resistance; MDR = antimicrobial resistance to three or more antimicrobial classes; [3]Hosmer and Lemeshow Goodness-of-fit; *P*-value; Covariate *P* values are from the * Likelihood-ratio test statistic. Significant set at $P < 0.0085$ (*Sidak-Bonferroni* correction).



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Highlights

- Antimicrobial and multi-drug resistance was common
- ESBL and plasmid-mediated AmpC-producing *E. coli* were uncommon.
- Most isolates were phylo-group B1; often antimicrobial resistant
- Phylo-group B2 isolates were more likely to be susceptible.
- The main risk for antimicrobial resistance was eating raw meat.